

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number  
**WO 01/23549 A1**

(51) International Patent Classification<sup>7</sup>: **C12N 15/12**,  
15/11, C07K 14/475, 14/51, 16/18, G01N 33/68

Floor, M Block, QEII Medical Centre, Nedlands, W.A.  
6009 (AU).

(21) International Application Number: PCT/AU00/01202

(74) Agent: **GRIFFITH HACK**; Level 3, 509 St Kilda Road,  
Melbourne, VIC 3004 (AU).

(22) International Filing Date:  
29 September 2000 (29.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
PQ 3147 29 September 1999 (29.09.1999) AU

(71) Applicant (*for all designated States except US*): **UNI-  
VERSITY OF WESTERN AUSTRALIA** [AU/AU];  
Nedlands, W.A. 6907 (AU).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **XU, Jiake** [CN/AU];  
Department of Surgery, Unit of Orthopaedics, The Uni-  
versity of Western Australia, 2nd Floor, M Block, QEII  
Medical Centre, Nedlands, W.A. 6009 (AU). **ZHENG,  
Ming-Hao** [CN/AU]; Department of Surgery, Unit of  
Orthopaedics, The University of Western Australia, 2nd

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *With international search report.*

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: **RAT OSTEOCLAST DIFFERENTIATION FACTOR**

(57) Abstract: The invention relates to an isolated nucleic acid encoding a rat osteoclast differentiation factor (ODF) or a biologi-  
cally active fragment thereof, the protein encoded by the said nucleic acid, expression vectors and host cells carrying the said nucleic  
acid.



**WO 01/23549 A1**

**Rat osteoclast differentiation factor**FIELD OF THE INVENTION

The present invention relates to polypeptides  
5 that are involved in osteoclast differentiation. More  
particularly, the invention relates to osteoclast  
differentiation factor (ODF). Most particularly, the  
present invention relates to ODF isolated from rats,  
nucleic acids encoding this protein, expression vectors and  
10 host cells for the reproduction of this protein.

BACKGROUND OF THE INVENTION

Bone formation and resorption are tightly  
controlled processors and are maintained in balance in  
15 healthy individuals; however, if an imbalance between the  
two processors does result then various diseases such as  
osteoporosis, osteopetrosis, Paget's disease and arthritis  
may occur. Primarily two cell types mediate these  
processors: osteoblasts, which secrete molecules that  
20 comprise the organic matrix of bone; and osteoclasts, which  
promote dissolution of the bone matrix and solubilisation  
of bone salts.

Osteoclasts are multinucleated giant cells that  
can resorb bone, and are formed from haematopoietic  
25 precursor cells by differentiation of monocyte/macrophage  
lineage cells. The growth and formation of mature  
functional osteoclasts from monocytes/macrophages is not  
well understood. Until recently, the early development of  
bone marrow precursor cells to preosteoclasts were believed  
30 to be mediated by soluble factors such as tumour necrosis  
factor- $\alpha$  (TNF- $\alpha$ ), TNF- $\beta$ , interleukin-1 (IL-1),  
interleukin-4 (IL-4), interleukin-6 (IL-6), and leukemia  
inhibitory factor (LIF). However, it has now been shown  
that a new member of the TNF receptor-ligand family has  
35 been discovered which has an effect on monocytes. This new  
member has been called osteoclast differentiation factor  
(ODF) or alternatively osteoprotegerin ligand (OPGL)/TNF-

related activation-induced cytokine (TRANCE)/receptor activator of NF- $\kappa$ B (RANK) ligand, and appears to be directly involved in the differentiation of monocytes/macrophages into osteoclasts (Lacey et al., 1998; 5 Yasuda et al., 1998; Wong et al., 1997; Anderson et al., 1997; PCT Application WO96/26272).

The membrane-bound and soluble forms of ODF have been shown to be sufficient to induce osteoclast formation *in vitro* (Lacey et al., 1998; Yasuda et al., 1998). It has 10 also been shown that mice with a disrupted ODF gene, lack osteoclasts, and suffer severe osteopetrosis and a defect in tooth eruption. These data indicate that ODF is an essential osteoclast differentiation factor *in vivo* (Kong et al., 1999). In addition to its role in 15 osteoclastogenesis, ODF has been shown to be a regulator of interactions between T-cells and dendritic cells *in vitro* (Wong et al., 1997; Anderson et al., 1997), and of lymph-node organogenesis and lymphocyte development *in vivo* (Kong et al., 1999). Further data which has recently been 20 obtained shows that ODF-induced osteoclastogenesis is mediated through RANK expressed on the cell surface of osteoclast progenitors (Nakagawa et al., 1998; Hsu et al., 1999). Transgenic mice expressing a soluble RANK-Fc fusion protein have reduced osteoclasts and display severe 25 osteopetrosis (Hsu et al., 1999). The activity of ODF-induced osteoclastogenesis is negatively regulated by a secreted OPGL or osteoclastogenesis inhibitor factor (OCIF) (Yasuda et al., 1998; Simonet et al., 1997).

While there has been a significant breakthrough 30 in recent times with the discovery of ODF, in particular the discovery of the human ODF binding factor as shown in PCT application WO98/46751, the entirety of which is incorporated herein by reference, there is a significant amount of data still to be elucidated. For example, in 35 WO98/46751 it is postulated that OPGL (ODF) may bind to a polypeptide factor involved in osteoclast differentiation and thereby block one or more terminal steps leading to the

formation of mature osteoclasts. These polypeptides may also play a role in osteoclast maturation and may be useful in the treatment of bone diseases. However, in order for these hypotheses to be confirmed, it is important that the molecular mechanisms by which ODF functions must be determined. Moreover, any identified polypeptides which affect ODF need to be studied in a suitable animal model that is predictive for metabolic diseases. Only in this way will studies on ODF/RANK/OPGL assist in our understanding of the molecular mechanisms of regulation of osteoclast bone resorption, and therefore the establishment of new ways to treat metabolic diseases. The use of these animal models will also assist in the analysis of any newly discovered polypeptides.

WO98/46751 does not disclose or teach towards such an animal model or an ODF that would function in such an animal model.

The use of the ovariectomized rat as the first choice animal model for the preclinical evaluation of agents used in the treatment and prevention of post or menopausal osteoporosis has been recommended by the US Federal Drug Administration (FDA). Given the biological significance of ODF, and the importance of the rat model of osteoporosis, it is suggested that the rat is the preferred animal model for the study of any polypeptide that is identified to interact with ODF.

Accordingly, the present invention attempts to overcome or at least alleviate some of the problems highlighted in the prior art. In particular, the present invention attempts to overcome or at least alleviate the problem associated with providing a useful animal model for the study of polypeptides that relate to ODF.

#### SUMMARY OF THE INVENTION

The present invention provides an isolated nucleic acid encoding a rat osteoclast differentiation factor (ODF) or biologically active fragment thereof.

Preferably, the nucleic acid is genomic DNA, cDNA, RNA, or hybrid molecule thereof. Most preferably, the nucleic acid is a DNA molecule having a nucleotide sequence as shown in Figure 1.

5 In a second aspect, the present invention provides a rat ODF polypeptide or biologically active fragment thereof. Preferably, the polypeptide has an amino acid sequence as shown in Figure 2.

10 Modified and variant forms of rat ODF may be produced *in vitro* by means of chemical or enzymatic treatment or *in vivo* by means of recombinant DNA technology. Such polypeptides may differ from native rat ODF, for example, by virtue of one or more amino acid substitutions, deletions or insertions, or in the extent or  
15 pattern of glycosylation, but substantially retain a biological activity of native rat ODF.

In a third aspect, the present invention provides a method of modulating the activity of cells comprising the step of administering to rat cells a protein encoded by the  
20 nucleic acid sequence shown in Figure 1. Preferably the activity modulated will be selected from the group consisting of cell proliferation, cell differentiation and cell viability.

In a fourth aspect, the present invention  
25 provides a process for the production of rat ODF comprising the steps of:

growing under suitable conditions a microorganism transformed or transfected with a nucleic acid which encodes said ODF; and

30 isolating the polypeptide product of the expression of said nucleic acid.

In a fifth aspect, the present invention provides an antisense nucleic acid that is capable of binding to the coding sequence of rat ODF. Preferably, the antisense is  
35 complementary to the nucleic acid as shown in Figure 1. More preferably, the antisense sequence will inhibit the activity of rat ODF in cells when transfected into them.

Even more preferably, the inhibition will be selected from the group consisting of cell proliferation, cell differentiation and cell viability.

In a sixth aspect, the present invention provides  
5 a fragment of rat ODF capable of eliciting an antibody which co-precipitates ligands that bind rat ODF.

In a seventh aspect, the present invention provides an antibody elicited by a rat ODF fragment according to the sixth aspect of the invention. Antibodies  
10 to rat ODF are produced by immunizing an animal with rat ODF or a fragment thereof, optionally in conjunction with an immunogenic polypeptide, and thereafter recovering antibodies from the serum of the immunized animals. Alternatively, monoclonal antibodies are prepared from  
15 cells of the immunized animal in conventional fashion. Immobilized anti-rat ODF antibodies are particularly useful in the detection of rat ODF in samples for research purposes, and in the purification of rat ODF. Accordingly, the antibody may be polyclonal or monoclonal, but is  
20 preferably monoclonal.

In an eighth aspect, the invention provides a polypeptide which is specifically co-precipitated by an antibody of the invention from a cell expressing full-length rat ODF protein. Preferably the cell is stably  
25 over-expressing the full-length rat ODF protein.

In a particularly preferred embodiment, the polypeptide has the ability to bind to rat ODF, and thereby to modulate an activity selected from the group consisting of cell cycle control, cellular differentiation and cell  
30 proliferation.

In an ninth aspect, the invention provides a method of screening for a ligand able to bind to and either activate or inhibit rat ODF. Such methods include but are not limited to:

- 35 a). use of antibodies to rat ODF to immunoprecipitate rat ODF and proteins bound to rat ODF;  
b). screening lambda phage expression libraries for

proteins that bind rat ODF peptides or fragments;

c). using cDNA sequences coding for specific extracellular and intracytoplasmic domains of rat ODF as "bait" sequences in the yeast two-hybrid system to screen  
5 for binding proteins;

d). using rat ODF peptides and/or fragments in solid-phase affinity binding assays such as chromatography and biosensor assays to identify proteins extracted from cells and tissues that bind to rat ODF peptides and fragments;

10 and

e). using monoclonal antibodies to rat ODF and/or fragments thereof to compete for binding of rat ODF;

f). using epitope labelled rat ODF fragment to screen for binding proteins in eukaryotic cell lysates.

15 In a tenth aspect, the present invention provides a method of assessing the ability of a candidate compound to bind to a rat ODF comprising the step of:

incubating the rat ODF with the candidate compound under conditions that allow binding; and

20 measuring the bound compound.

In an eleventh aspect, the present invention provides a method of assessing the ability of a test compound to increase or decrease binding of an ODF to an ODF receptor comprising the steps of:

25 incubating ODF, ODF receptor and optionally the test compound under conditions that allow binding of the ODF to ODF receptor; and

measuring the binding of ODF to ODF receptor in the presence and absence of the test compound.

30 In further aspects, the invention provides a method for determining the presence of a nucleic acid molecule encoding rat ODF in test samples prepared from cells, tissues, or biological fluids, comprising contacting the test sample with isolated DNA comprising all or a  
35 portion of the nucleotide coding sequence for rat ODF and determining whether the isolated DNA hybridizes to a nucleic acid molecule in the test sample. DNA comprising

all or a portion of the nucleotide coding sequence for rat ODF is also used in hybridization assays to identify and to isolate nucleic acids sharing substantial sequence identity to the coding sequence for rat ODF, such as nucleic acids  
5 that encode allelic variants of rat ODF.

Also provided is a method for amplifying a nucleic acid molecule encoding rat ODF that is present in a test sample, comprising the use of oligonucleotides having a portion of the nucleotide coding sequence for rat ODF as  
10 primers in a polymerase chain reaction.

Accordingly, the present invention provides molecules capable of binding to rat ODF.

Preferably, the molecules are either ligands or antibodies, or functional fragments thereof. Where the  
15 molecule is an antibody it is preferable that the antibody is an antagonist or an agonist of rat ODF.

It is contemplated that by using the polypeptides of the invention, or an agonist or antagonist thereof, it will be possible to effect a number of interventions into  
20 cell growth and differentiation.

Rat ODF, its derivatives, or its antibodies may also be formulated with physiologically acceptable carriers, especially for *in vivo* use.

Throughout the description and claims of this  
25 specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", means "including but not limited to", and is not intended to exclude other additives, components, integers or steps".

### 30 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence of rat ODF.

Figure 2 shows the amino acid sequence of rat ODF.

35 Figure 3 shows the multiple alignment of the human, mouse, and rat ODF protein sequences.

Figure 4 shows the tissue distribution of ODF



transcripts.

Figure 5 shows ODF induction of osteoclastogenesis.

Figure 6 shows ODF induction of systemic hypercalcemia. A graph of the serum calcium levels in ODF or GST injected newborn rats.

Figure 7 shows ODF induction of osteoclast polarization *in vivo*.

Figure 8 shows a graph indicating the percentage of polarized osteoclasts in ODF or GST injected newborn rats.

Figure 9 shows the induction of systemic hypercalcaemia *in vivo* and osteoclast polarization *ex vivo* by the TNF-like core region of rODF. (A) shows the comparison of serum calcium levels in new born rats injected with either GST-rODF (aa160-318) or GST alone (control). (B) shows the F-actin ring formation in osteoclasts. (C) shows the comparison of percentages of polarized osteoclasts (F-actin ring) in new born rats injected with either GST-rODF (aa160-318) or GST (control). (D) shows the histomorphometry analysis of tibiae in rats injected with either GST-rODF (aa160-318) or GST. The total numbers of osteoclasts/bone perimeter and osteoclast resorbing surfaces/bone perimeters were determined. #  $p > 0.05$  \*  $p < 0.05$  \*\*  $p < 0.01$ .

#### ABBREVIATIONS AND TERMS USED

Unless indicated otherwise the following terms and abbreviations have the following meanings throughout the specification and claims. In the sequence listings standard abbreviations for nucleotides are used. These are:

a	a	adenine
g	g	guanine
c	c	cytosine
35 t	t	thymine
u	u	uracil
r	g or a	purine

- 9 -

	y	t/u or c	pyrimidine
	m	a or c	amino
	k	g or t/u	keto
	s	g or c	strong interactions <sup>3</sup> H-bonds
5	w	a or t/u	weak interactions <sup>2</sup> H-bonds
	b		unknown, or any other.

Unless stated otherwise, where modified bases are used these are represented as follows:

	ac4c	4-acetylcytidine
10	chm5u	5-(carboxyhydroxymethyl)uridine
	cm	2'-O-methylcytidine
	cmnm5s2u	5-carboxymethylaminomethyl-2-thiouridine
	cmnm5u	5-carboxymethylaminomethyluridine
	d	dihydrouridine
15	fm	2'-O-methylpseudouridine
	gal q beta	D-galactosylqueuosine
	gm	2'-O-methylguanosine
	I	inosine
	i6a	N6-isopentenyladenosine
20	m1a	1-methyladenosine
	m1f	1-methylpseudouridine
	m1g	1-methylguanosine
	m1I	1-methylinosine
	m22g	2,2-dimethylguanosine
25	m2a	2-methyladenosine
	m2g	2-methylguanosine
	m3c	3-methylcytidine
	m5c	5-methylcytidine
	m6a	N6-methyladenosine
30	m7g	7-methylguanosine
	mam5u	5-methylaminomethyluridine
	mam5s2u	5-methoxyaminomethyl-2-thiouridine
	man q beta	D-mannosylqueuosine
	mcm5s2u	5-methoxycarbonylmethyl-2-thiouridine
35	mcm5u	5-methoxycarbonylmethyluridine
	mo5u	5-methoxyuridine
	ms2i6a	2-methylthio-N6-isopentenyladenosine

- 10 -

	ms2t6a	N-((9-beta-D-ribofuranosyl-2-methyl-thiopurine-6-yl) carbamoyl) threonine
	mt6a	N-((9-beta-D-ribofuranosylpurine-6-yl)N-methylcarbamoyl) threonine
5	mv	uridine-5-oxyacetic acid-methylester
	o5u	uridine-5-oxyacetic acid
	osyw	wybutoxosine
	p	pseudouridine
	q	queuosine
10	s2t	5-methyl-2-thiouridine
	s2c	2-thiocytidine
	s2t	5-methyl-2-thiouridine
	s2u	2-thiouridine
	s4u	4-thiouridine
15	t	5-methyluridine
	t6a	N-((9-beta-D-ribofuranosylpurine-6-yl)-carbamoyl) threonine
	tm	2'-O-methyl-5-methyluridine
	um	2'-O-methyluridine
20	yw	wybutosine
	x	3-(3-amino-3-carboxy-propyl)uridine.

The following three and single letter codes are used for amino acids:

	Ala	A	Alanine
25	Arg	R	Arginine
	Asn	N	Asparagine
	Asp	D	Aspartic Acid
	Asx	B	Asp or Asn
	Cys	C	Cysteine
30	Gln	Q	Glutamine
	Glu	E	Glutamic Acid
	Glx	Z	Glu or Gln
	Gly	G	Glycine
	His	H	Histidine
35	Ile	I	Isoleucine
	Leu	L	Leucine
	Lys	K	Lysine

- 11 -

	Met	M	Methionine
	Phe	F	Phenylalanine
	Pro	P	Proline
	Ser	S	Serine
5	Thr	T	Threonine
	Trp	W	Tryptophan
	Tyr	Y	Tyrosine
	Val	V	Valine
	Xaa	-	unknown or other

10           It will be appreciated by those skilled in the art that modified amino acids may be used as a replacement for naturally occurring amino acids. The following modified or unusual amino acid are anticipated as being useful in the present invention:

15	Aad	2-Aminoadipic acid
	bAad	3-Aminoadipic acid
	bAla	beta-Alanine, beta-Amino propionic acid
	Abu	2-Aminobutyric acid
	4Abu	4-Aminobutyric acid, piperidinic acid
20	Acp	6-Aminocaproic acid
	Ahe	2-Aminoheptanoic acid
	Aib	2-Aminoisobutyric acid
	BAib	3-Aminoisobutyric acid
	Apm	2-Aminopimelic acid
25	Dbu	2,4-Diaminobutyric acid
	Des	Desmosine
	Dpm	2,2' -Diaminopimelic acid
	Dpr	2,3-Diaminopropionic acid
	EtGly	N-Ethylglycine
30	EtAsn	N-Ethylasparagine
	Hyl	Hydroxylysine
	AHyl	allo-Hydroxylysine
	3Hyp	3-Hydroxyproline
	4Hyp	4-Hydroxyproline
35	Ide	Isodesmosine
	Aile	allo-Isoleucine
	MeGly	N-Methylglycine, sarcosine

	MeIle	N-Methylisoleucine
	MeLys	6-N-Methyllysine
	MeVal	N-Methylvaline
	Nva	Norvaline
5	Nle	Norleucine
	Orn	Ornithine

Unless stated otherwise the following features are understood to be part of the present invention:

Attenuator means either (1) region of DNA at  
10 which regulation of termination of transcription occurs, which controls the expression of some bacterial operons; or (2) sequence segment located between the promoter and the first structural gene that causes partial termination of transcription.

15 C-region means constant region of immunoglobulin light and heavy chains, and T-cell receptor alpha, beta, and gamma chains; includes one or more exons depending on the particular chain.

CAAT-signal or CAAT box means a part of a  
20 conserved sequence located about 75 bp up-stream of the start point of eukaryotic transcription units which maybe involved in RNA polymerase binding.

iDNA means intervening DNA, which is DNA that is eliminated through any of several kinds of recombination  
25 Intron means a segment of DNA that is transcribed, but removed from within the transcript by splicing together the sequences (exons) on either side of it.

mat-peptide means mature peptide or protein coding sequence; coding sequence for the mature or final  
30 peptide or protein product following post-translational modification; the location does not include the stop codon (unlike the corresponding CDS).

misc-signal means any region containing a signal controlling or altering gene function or expression that  
35 cannot be described by other Signal keys (promoter, CAAT-signal, TATA-signal, -35-signal, -10-signal, GC-signal, RBS, polyA-signal, enhancer, attenuator, terminator, and

rep-origin).

A modified-base indicates nucleotide is a modified nucleotide and should be substituted for by the indicated molecule (given in the mod-base qualifier value)  
5 mRNA is messenger RNA and includes 5' untranslated region (5'UTR), coding sequences (CDS, exon) and 3' untranslated region (3'UTR).

Mutation means a related strain has an abrupt, inheritable change in the sequence at this location.

10 Rat ODF or rat ODF protein refers to a polypeptide or protein encoded by the rat ODF nucleotide sequence set forth in Figure 1; a polypeptide that is the translated amino acid sequence set forth in Figure 2; fragments thereof having greater than about 5 amino acid  
15 residues and comprising an immune epitope or other biologically active site of rat ODF; amino acid sequence variants of the amino acid sequence set forth in Figure 2 wherein one or more amino acid residues are added at the N- or C-terminus of, or within, said Figure 2 sequence or its  
20 fragments as defined above; amino acid sequence variants of said Figure 2 sequence or its fragments as defined above wherein one or more amino acid residues of said Figure 2 sequence or fragment thereof are deleted, and optionally substituted by one or more amino acid residues; and  
25 derivatives of the above proteins, polypeptides, or fragments thereof, wherein an amino acid residue has been covalently modified so that the resulting product is a non-naturally occurring amino acid.

Rat ODF amino acid sequence variants may be made  
30 synthetically, for example, by site-directed or PCR mutagenesis, or may exist naturally, as in the case of allelic forms and other naturally occurring variants of the translated amino acid sequence set forth in Figure 2 that may occur in other rodent species. In any event, such  
35 fragments, variants, and derivatives exclude any polypeptide heretofore identified, including any known ODF, as well as statutorily obvious variants thereof.

A rat ODF amino acid sequence variant is included within the scope of the invention provided that it is functionally active. As used herein, "functionally active" and "functional activity" in reference to rat ODF means  
5 that the rat ODF is able to promote or enhance the differentiation of rat osteoclasts, and/or that the rat ODF is immunologically cross-reactive with an antibody directed against an epitope of naturally occurring rat ODF.

Therefore, rat ODF amino acid sequence variants  
10 generally will share at least about 75% (preferably greater than 80% and more preferably greater than 90%) sequence identity with the translated nucleotide sequence set forth in Figure 1, after aligning the sequences to provide for maximum homology, as determined, for example, by the Fitch,  
15 et al., *Proc. Nat. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman, et al., *J. Mol. Biol.* 48:443-453 (1970).

Amino acid sequence variants of rat ODF are prepared by introducing appropriate nucleotide changes into  
20 the rat ODF DNA and thereafter expressing the resulting modified DNA in a host cell, or by *in vitro* synthesis. Such variants include, for example, deletions from, or insertions or substitutions of, amino acid residues within the rat ODF amino acid sequence set forth in Figure 2. Any  
25 combination of deletion, insertion, and substitution may be made to arrive at an amino acid sequence variant of rat ODF, provided that such variant possesses the desired characteristics described herein. Changes that are made in the amino acid sequence set forth in Figure 2 to arrive at  
30 an amino acid sequence variant of rat ODF also may result in further modifications of the rat ODF upon its expression in host cells, for example, by virtue of such changes introducing or moving sites of glycosylation, or introducing membrane anchor sequences as described, for  
35 example, in PCT Pat. Pub. No. WO 89/01041 (published February 9, 1989).

There are two principal variables in the

construction of amino acid sequence variants of rat ODF:  
the location of the mutation site and the nature of the  
mutation. These are variants from the amino acid sequence  
set forth in Figure 2, and may represent naturally  
5 occurring allelic forms of rat ODF, or predetermined mutant  
forms of rat ODF made by mutating rat ODF DNA, either to  
arrive at an allele or a variant not found in nature. In  
general, the location and nature of the mutation chosen  
will depend upon the rat ODF characteristic to be modified.  
10 For example, due to the degeneracy of nucleotide  
coding sequences, mutations can be made in the rat ODF  
nucleotide sequence set forth in Figure 1 without affecting  
the amino acid sequence of the rat ODF encoded thereby.  
Other mutations can be made that will result in a rat ODF  
15 that has an amino acid sequence different from that set  
forth in Figure 2, but which is functionally active. Such  
functionally active amino acid sequence variants of rat ODF  
are selected, for example, by substituting one or more  
amino acid residues in the amino acid sequence set forth in  
20 Figure 2 with other amino acid residues of a similar or  
different polarity or charge.

One useful approach is called "alanine scanning  
mutagenesis." Here, an amino acid residue or group of  
target residues are identified (e.g., charged residues such  
25 as arg, asp, his, lys, and glu) and, by means of  
recombinant DNA technology, replaced by a neutral or  
negatively charged amino acid (most preferably alanine or  
polyalanine) to affect the interaction of the amino acids  
with the surrounding aqueous environment in or outside the  
30 cell. Cunningham, et al., Science 244: 1081-1085 (1989).  
Those domains demonstrating functional sensitivity to the  
substitutions then are refined by introducing further or  
other variants at or for the sites of substitution.

Obviously, such variations that, for example,  
35 convert the amino acid sequence set forth in Figure 2 to  
the amino acid sequence of a known ODF, such as mouse ODF  
or human ODF, or another known polypeptide or protein are



not included within the scope of this invention, nor are any other fragments, variants, and derivatives of the rat ODF amino acid sequence that are not novel and unobvious over the prior art. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed rat ODF variants are screened for functional activity.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions from regions of substantial homology with other ODF ligands, for example, are more likely to affect the functional activity of rat ODF. Generally, the number of consecutive deletions will be selected so as to preserve the tertiary structure of the rat ODF in the affected domain, e.g., beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one amino acid residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions made within the amino acid sequence set forth in Figure 2) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include rat ODF with an N-terminal methionyl residue (such as may result from the direct expression of rat ODF in recombinant cell culture), and rat ODF with a heterologous N-terminal signal sequence to improve the secretion of rat ODF from recombinant host cells. Such signal sequences generally will be homologous to the host cell used for expression of rat ODF, and include STII or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertions include the fusion to

the N- or C-terminus of rat ODF of immunogenic polypeptides (for example, bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein), and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions, albumin, or ferritin, as described in PCT Pat. Pub. No. WO 89/02922 (published April 6, 1989).

The third group of variants are those in which at least one amino acid residue in the amino acid sequence set forth in Figure 2, preferably one to four, more preferably one to three, even more preferably one to two, and most preferably only one, has been removed and a different residue inserted in its place. The sites of greatest interest for making such substitutions are in the regions of the amino acid sequence set forth in Figure 2 that have the greatest homology with other ODF's. Those sites are likely to be important to the functional activity of the rat ODF. Accordingly, to retain functional activity, those sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner.

Insertional, deletional, and substitutional changes in the amino acid sequence set forth in Figure 2 may be made to improve the stability of rat ODF. For example, trypsin or other protease cleavage sites are identified by inspection of the encoded amino acid sequence for an arginyl or lysinyl residue. These are rendered inactive to protease by substituting the residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue. Also, any cysteine residues not involved in maintaining the proper conformation of rat ODF for functional activity may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Covalent modifications of rat ODF molecules also

are included within the scope of this invention. For example, covalent modifications are introduced into rat ODF by reacting targeted amino acid residues of the rat ODF with an organic derivatizing agent that is capable of  
5 reacting with selected amino acid side chains or the N- or C-terminal residues.

Cysteiny l residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl  
10 or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-  
15 chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-  
20 bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0. Lysiny l and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of  
25 reversing the charge of the lysiny l residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-  
30 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginy l residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and  
35 ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high  $pK_a$  of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group. The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels  
5 into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using  $^{125}\text{I}$  or  
10  $^{131}\text{I}$  to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ( $\text{R}'\text{-N}=\text{C}=\text{N-R}$ ), where R and R' are different alkyl groups, such  
15 as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyll and glutaminyll residues by reaction with  
20 ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking rat ODF to a water-insoluble support matrix or surface for use in the method for purifying anti-rat ODF antibodies, or for therapeutic use. Commonly used  
25 crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and  
30 bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices  
35 such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440

are employed for protein immobilization.

Glutaminy and asparaginy residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these  
5 residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or  
10 threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. Creighton, Proteins: Structure and Molecular Properties, pp.79-86 (W.H. Freeman & Co., 1983).  
15 Rat ODF may also be covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,179,337; 4,301,144; 4,496,689; 4,640,835; 4,670,417; or 4,791,192.

20 "Rat ODF antagonist" or "antagonist" refers to a substance that opposes or interferes with a functional activity of rat ODF.

"Cell," "host cell," "cell line," and "cell culture" are used interchangeably and all such terms should  
25 be understood to include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of times the cultures have been passaged. It should also be understood that all progeny may not be  
30 precisely identical in DNA content, due to deliberate or inadvertent mutations.

"Plasmids" are DNA molecules that are capable of replicating within a host cell, either extrachromosomally or as part of the host cell chromosome(s), and are  
35 designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available

- 21 -

on an unrestricted basis, or can be constructed from such available plasmids as disclosed herein and/or in accordance with published procedures. In certain instances, as will be apparent to the ordinarily skilled artisan, other  
5 plasmids known in the art may be used interchangeably with plasmids described herein.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked nucleotide coding sequence in a particular host cell. The  
10 control sequences that are suitable for expression in prokaryotes, for example, include origins of replication, promoters, ribosome binding sites, and transcription termination sites. The control sequences that are suitable for expression in eukaryotes, for example, include origins  
15 of replication, promoters, ribosome binding sites, polyadenylation signals, and enhancers.

An "exogenous" element is one that is foreign to the host cell, or homologous to the host cell but in a position within the host cell in which the element is  
20 ordinarily not found.

"Digestion" of DNA refers to the catalytic cleavage of DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes or restriction endonucleases, and the sites within  
25 DNA where such enzymes cleave are called restriction sites. If there are multiple restriction sites within the DNA, digestion will produce two or more linearized DNA fragments (restriction fragments). The various restriction enzymes used herein are commercially available and their reaction  
30 conditions, cofactors, and other requirements as established by the enzyme manufacturers are used.

Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the micro-organism from which  
35 each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1  $\mu$ g of DNA is digested with about 1-2 units of

enzyme in about 20  $\mu$ l of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer, and/or are well known in the art.

5               "Recovery" or "isolation" of a given fragment of DNA from a restriction digest typically is accomplished by separating the digestion products, which are referred to as "restriction fragments" on a polyacrylamide or agarose gel by electrophoresis, identifying the fragment of interest on  
10 the basis of its mobility relative to that of marker DNA fragments of known molecular weight, excising the portion of the gel that contains the desired fragment, and separating the DNA from the gel, for example by electroelution.

15               "Ligation" refers to the process of forming phospho-diester bonds between two double-stranded DNA fragments. Unless otherwise specified, ligation is accomplished using known buffers and conditions with 10 units of T4 DNA ligase per 0.5  $\mu$ g of approximately  
20 equimolar amounts of the DNA fragments to be ligated.

              "Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (involving, for example, triester, phosphoramidite, or phosphonate chemistry), such  
25 as described by Engels, et al., *Agnew. Chem. Int. Ed. Engl.* 28:716-734 (1989). They are then purified, for example, by polyacrylamide gel electrophoresis.

              "Polymerase chain reaction," or "PCR," as used herein generally refers to a method for amplification of a  
30 desired nucleotide sequence *in vitro*, as described in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using two oligonucleotide primers capable of hybridizing preferentially to a template nucleic acid. Typically, the  
35 primers used in the PCR method will be complementary to nucleotide sequences within the template at both ends of or flanking the nucleotide sequence to be amplified, although

primers complementary to the nucleotide sequence to be amplified also may be used. Wang, et al., in PCR Protocols, pp.70-75 (Academic Press, 1990); Ochman, et al., in PCR Protocols, pp. 219-227; Triglia, et al., *Nuc. Acids Res.* 16:8186 (1988).

"PCR cloning" refers to the use of the PCR method to amplify a specific desired nucleotide sequence that is present amongst the nucleic acids from a suitable cell or tissue source, including total genomic DNA and cDNA transcribed from total cellular RNA. Frohman, et al., *Proc. Nat. Acad. Sci. USA* 85:8998-9002 (1988); Saiki, et al., *Science* 239:487-492 (1988); Mullis, et al., *Meth. Enzymol.* 155:335-350 (1987).

"Stringent conditions" for hybridization or annealing of nucleic acid molecules are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

"Rat ODF nucleic acid" is RNA or DNA that encodes rat ODF. "Rat ODF DNA" is DNA that encodes rat ODF. Rat ODF DNA is obtained from cDNA or genomic DNA libraries, or by *in vitro* synthesis. Identification of rat ODF DNA within a cDNA or a genomic DNA library, or in some other mixture of various DNAs, is conveniently accomplished by the use of an oligonucleotide hybridization probe that is labeled with a detectable moiety, such as a radioisotope. Keller, et al., *DNA Probes*, pp.149-213 (Stockton Press,



1989). To identify DNA encoding rat ODF, the nucleotide sequence of the hybridization probe preferably is selected so that the hybridization probe is capable of hybridizing preferentially to DNA encoding the rat ODF amino acid sequence set forth in Figure 2, or a variant or derivative thereof as described herein, under the hybridization conditions chosen. Another method for obtaining rat ODF nucleic acid is to chemically synthesize it using one of the methods described, for example, by Engels, et al.,  
5  
10 *Agnew. Chem. Int. Ed. Engl.* 28:716-734 (1989).

If the entire nucleotide coding sequence for rat ODF is not obtained in a single cDNA, genomic DNA, or other DNA, as determined, for example, by DNA sequencing or restriction endonuclease analysis, then appropriate DNA fragments (e.g., restriction fragments or PCR amplification products) may be recovered from several DNAs and covalently joined to one another to construct the entire coding sequence. The preferred means of covalently joining DNA fragments is by ligation using a DNA ligase enzyme, such as  
15  
20 T4 DNA ligase.

"Isolated" rat ODF nucleic acid is rat ODF nucleic acid that is identified and separated from (or otherwise substantially free from), contaminant nucleic acid encoding other polypeptides. The isolated rat ODF nucleic acid can be incorporated into a plasmid or expression vector, or can be labeled for diagnostic and probe purposes, using a label as described further herein in the discussion of diagnostic assays and nucleic acid hybridization methods.  
25

For example, isolated rat ODF DNA, or a fragment thereof comprising at least about 15 nucleotides, is used as a hybridization probe to detect, diagnose, or monitor disorders or diseases that involve changes in rat ODF expression, such as may result from neuron damage. In one embodiment of the invention, total RNA in a tissue sample from a patient (that is, a human or other mammal) can be assayed for the presence of rat ODF messenger RNA, wherein  
30  
35

the decrease in the amount of rat ODF messenger RNA is indicative of neuronal degeneration.

Isolated rat ODF nucleic acid also is used to produce rat ODF by recombinant DNA and recombinant cell culture methods. In various embodiments of the invention, host cells are transformed or transfected with recombinant DNA molecules comprising an isolated rat ODF DNA, to obtain expression of the rat ODF DNA and thus the production of rat ODF in large quantities. DNA encoding amino acid sequence variants of rat ODF is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants of rat ODF) or preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding a variant or a non-variant form of rat ODF.

Site-directed mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of rat ODF DNA. This technique is well known in the art, Zoller, et al., *Meth. Enz.* 100:4668-500 (1983); Zoller, et al., *Meth. Enz.* 154:329-350 (1987); Carter, *Meth. Enz.* 154:382-403 (1987); Horwitz, et al., *Meth. Enz.* 185:599-611 (1990), and has been used, for example, to produce amino acid sequence variants of trypsin and T4 lysozyme, which variants have certain desired functional properties. Perry, et al., *Science* 226:555-557 (1984); Craik, et al., *Science* 228:291-297 (1985).

Briefly, in carrying out site-directed mutagenesis of rat ODF DNA, the rat ODF DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such rat ODF DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of rat ODF DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded

DNA.

Oligonucleotides for use as hybridization probes or primers may be prepared by any suitable method, such as by purification of a naturally occurring DNA or by *in vitro* synthesis. For example, oligonucleotides are readily  
5 synthesized using various techniques in organic chemistry, such as described by Narang, et al., *Meth. Enzymol.* 68:90-98 (1979); Brown, et al., *Meth. Enzymol.* 68:109-151 (1979); Caruther, et al., *Meth. Enzymol.* 154:287-313  
10 (1985). The general approach to selecting a suitable hybridization probe or primer is well known. Keller, et al., *DNA Probes*, pp.11-18 (Stockton Press, 1989). Typically, the hybridization probe or primer will contain 10-25 or more nucleotides, and will include at least 5  
15 nucleotides on either side of the sequence encoding the desired mutation so as to ensure that the oligonucleotide will hybridize preferentially to the single-stranded DNA template molecule.

Multiple mutations are introduced into rat ODF  
20 DNA to produce amino acid sequence variants of rat ODF comprising several or a combination of insertions, deletions, or substitutions of amino acid residues as compared to the amino acid sequence set forth in Figure 2. If the sites to be mutated are located close together, the  
25 mutations may be introduced simultaneously using a single oligonucleotide that encodes all of the desired mutations. If, however, the sites to be mutated are located some distance from each other (separated by more than about ten nucleotides), it is more difficult to generate a single  
30 oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each desired mutation. The  
oligonucleotides are then annealed to the single-stranded  
35 template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for introducing a single mutation: a single strand of a previously prepared rat ODF DNA is used as a template, an oligonucleotide encoding the first desired mutation is annealed to this template, and a heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid sequence variants of rat ODF. Higuchi, in *PCR Protocols*, pp.177-183 (Academic Press, 1990); Vallette, et al., *Nuc. Acids Res.* 17:723-733 (1989). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, for example, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a nucleotide sequence within the opposite strand of the plasmid DNA, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer

pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone. Wagner, et al., in PCR Topics, pp.69-71 (Springer-Verlag, 1991).  
5 If the ratio of template to product amplified DNA is extremely low, the majority of product DNA fragments incorporate the desired mutation(s). This product DNA is used to replace the corresponding region in the plasmid  
10 that served as PCR template using standard recombinant DNA methods. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to  
15 the plasmid fragment in a three (or more)-part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al., *Gene*, 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the rat ODF DNA to  
20 be mutated. The codon(s) in the rat ODF DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-  
25 mediated mutagenesis method to introduce them at appropriate locations in the rat ODF DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired  
30 mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed  
35 to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the

mutated rat ODF DNA sequence.

Rat ODF DNA, whether cDNA or genomic DNA or a product of *in vitro* synthesis, is ligated into a replicable vector for further cloning or for expression. "Vectors" are plasmids and other DNAs that are capable of replicating autonomously within a host cell, and as such, are useful for performing two functions in conjunction with compatible host cells (a vector-host system). One function is to facilitate the cloning of the nucleic acid that encodes the rat ODF, i.e., to produce usable quantities of the nucleic acid. The other function is to direct the expression of rat ODF. One or both of these functions are performed by the vector-host system. The vectors will contain different components depending upon the function they are to perform as well as the host cell with which they are to be used for cloning or expression.

To produce rat ODF, an expression vector will contain nucleic acid that encodes rat ODF as described above. The rat ODF of this invention is expressed directly in recombinant cell culture, or as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the junction between the heterologous polypeptide and the rat ODF.

In one example of recombinant host cell expression, mammalian cells are transfected with an expression vector comprising rat ODF DNA and the rat ODF encoded thereby is recovered from the culture medium in which the recombinant host cells are grown. But the expression vectors and methods disclosed herein are suitable for use over a wide range of prokaryotic and eukaryotic organisms.

Prokaryotes may be used for the initial cloning of DNAs and the construction of the vectors useful in the invention. However, prokaryotes may also be used for expression of DNA encoding rat ODF. Polypeptides that are produced in prokaryotic host cells typically will be non-

glycosylated.

Plasmid or viral vectors containing replication origins and other control sequences that are derived from species compatible with the host cell are used in connection with prokaryotic host cells, for cloning or expression of an isolated DNA. For example, *E. coli* typically is transformed using pBR322, a plasmid derived from an *E. coli* species. Bolivar, et al., *Gene* 2:95-113 (1987). PBR322 contains genes for ampicillin and tetracycline resistance so that cells transformed by the plasmid can easily be identified or selected. For it to serve as an expression vector, the pBR322 plasmid, or other plasmid or viral vector, must also contain, or be modified to contain, a promoter that functions in the host cell to provide messenger RNA (mRNA) transcripts of a DNA inserted downstream of the promoter. Rangagwala, et al., *Bio/Technology* 9:477-479 (1991).

In addition to prokaryotes, eukaryotic microbes, such as yeast, may also be used as hosts for the cloning or expression of DNAs useful in the invention. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used eukaryotic microorganism. Plasmids useful for cloning or expression in yeast cells of a desired DNA are well known, as are various promoters that function in yeast cells to produce mRNA transcripts.

Furthermore, cells derived from multicellular organisms also may be used as hosts for the cloning or expression of DNAs useful in the invention. Mammalian cells are most commonly used, and the procedures for maintaining or propagating such cells *in vitro*, which procedures are commonly referred to as tissue culture, are well known. Kruse & Patterson, eds., *Tissue Culture* (Academic Press, 1977). Examples of useful mammalian cells are human cell lines such as 293, HeLa, and WI-38, monkey cell lines such as COS-7 and VERO, and hamster cell lines such as BHK-21 and CHO, all of which are publicly available from the American Type Culture Collection (ATCC),

Rockville, Maryland 20852 USA.

Expression vectors, unlike cloning vectors, should contain a promoter that is recognized by the host organism and is operably linked to the rat ODF nucleic acid. Promoters are untranslated sequences that are located upstream from the start codon of a gene and that control transcription of the gene (that is, the synthesis of mRNA). Promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate high level transcription of the DNA under their control in response to some change in culture conditions, for example, the presence or absence of a nutrient or a change in temperature.

A large number of promoters are known, that may be operably linked to rat ODF DNA to achieve expression of rat ODF in a host cell. This is not to say that the promoter associated with naturally occurring rat ODF DNA is not usable. However, heterologous promoters generally will result in greater transcription and higher yields of expressed rat ODF.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoters, Goeddel, et al., *Nature* 281:544-548 (1979), tryptophan (trp) promoter, Goeddel, et al., *Nuc. Acids Res.* 8:4057-4074 (1980), and hybrid promoters such as the tac promoter, deBoer, et al., *Proc. Natl. Acad. Sci. USA* 80:21-25 (1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, Siebenlist, et al., *Cell* 20:269-281 (1980), thereby enabling a skilled worker operably to ligate them to DNA encoding rat ODF using linkers or adaptors to supply any required restriction sites. Wu, et al., *Meth. Enz.* 152:343-349 (1987).

Suitable promoters for use with yeast hosts include the promoters for 3-phosphoglycerate kinase, Hitzeman, et al., *J. Biol. Chem.* 255:12073-12080 (1980); Kingsman, et al., *Meth. Enz.* 185:329-341 (1990), or other glycolytic enzymes such as enolase, glyceraldehyde-3-phos-



phate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

- 5 Dodson, et al., *Nuc. Acids res.* 10:2625-2637 (1982); Emr, *Meth. Enz.* 185:231-279 (1990).

Expression vectors useful in mammalian cells typically include a promoter derived from a virus. For example, promoters derived from polyoma virus, adenovirus, 10 cytomegalovirus (CMV), and simian virus 40 (SV40) are commonly used. Further, it is also possible, and often desirable, to utilize promoter or other control sequences associated with a naturally occurring DNA that encodes rat ODF, provided that such control sequences are functional in 15 the particular host cell used for recombinant DNA expression.

Other control sequences that are desirable in an expression vector in addition to a promoter are a ribosome binding site, and in the case of an expression vector used 20 with eukaryotic host cells, an enhancer. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase the level of transcription. Many enhancer sequences are now known from mammalian genes (for example, the genes for globin, 25 elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, the enhancer used will be one from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma 30 enhancer on the late side of the replication origin, and adenovirus enhancers. Kriegler, *Meth. Enz.* 185:512-527 (1990).

Expression vectors may also contain sequences necessary for the termination of transcription and for 35 stabilizing the messenger RNA (mRNA). Balbas, et al., *Meth. Enz.* 185:14-37 (1990); Levinson, *Meth. Enz.* 185:485-511 (1990). In the case of expression vectors used with

eukaryotic host cells, such transcription termination sequences may be obtained from the untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain polyadenylation sites as well as transcription termination  
5 sites. Birnsteil, et al., *Cell* 41:349-359 (1985).

In general, control sequences are DNA sequences necessary for the expression of an operably linked coding sequence in a particular host cell. "Expression" refers to transcription and/or translation. "Operably linked" refers  
10 to the covalent joining of two or more DNA sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, DNA for a presequence or secretory leader is operably  
15 linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to  
20 a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase.

Linking is accomplished by ligation at convenient  
25 restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods. Expression and cloning vectors also will contain a sequence that enables the vector to replicate in one or more  
30 selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosome(s), and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of  
35 bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and

various viral origins (for example, from SV40, polyoma, or adenovirus) are useful for cloning vectors in mammalian cells. Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector may be cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

The expression vector may also include an amplifiable gene, such as that comprising the coding sequence for dihydrofolate reductase (DHFR). Cells containing an expression vector that includes a DHFR gene may be cultured in the presence of methotrexate, a competitive antagonist of DHFR. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA sequences comprising the expression vector, Ringold, et al., *J. Mol. Biol. Genet.* 1:165-175 (1981), such as a DNA sequence encoding rat ODF. In that manner, the level of rat ODF produced by the cells may be increased.

DHFR protein encoded by the expression vector also may be used as a selectable marker of successful transfection. For example, if the host cell prior to transformation is lacking in DHFR activity, successful transformation by an expression vector comprising DNA sequences encoding rat ODF and DHFR protein can be determined by cell growth in medium containing methotrexate. Also, mammalian cells transformed by an expression vector comprising DNA sequences encoding rat ODF, DHFR protein, and aminoglycoside 3' phosphotransferase (APH) can be determined by cell growth in medium containing an aminoglycoside antibiotic such as kanamycin or neomycin. Because eukaryotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as *neo<sup>r</sup>* genes, may be used as dominant

selectable markers in a wide range of eukaryotic host cells, by which cells transfected by the vector can easily be identified or selected. Jiminez, et al., *Nature*, 287:869-871 (1980); Colbere-Garapin, et al., *J. Mol. Biol.* 150:1-14 (1981); Okayama & Berg, *Mol. Cell. Biol.*, 3:280-289 (1983).

Many other selectable markers are known that may be used for identifying and isolating recombinant host cells that express rat ODF. For example, a suitable selection marker for use in yeast is the *trp1* gene present in the yeast plasmid YRp7. Stinchcomb, et al., *Nature* 282:39-43 (1979); Kingsman, et al., *Gene* 7:141-152 (1979); Tschemper, et al., *Gene* 10:157-166 (1980). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (available from the American Type Culture Collection, Rockville, Maryland 20852 USA). Jones, *Genetics* 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC Nos. 20622 or 38626) are complemented by known plasmids bearing the *Leu2* gene.

Particularly useful in the invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding rat ODF. In general, transient expression involves the use of an expression vector that is able to efficiently replicate in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Yang, et al., *Cell* 47:3-10

(1986); Wong, et al., *Science* 228:810-815 (1985); Lee, et al., *Proc. Nat Acad. Sci. USA* 82:4360-4364 (1985). Thus, transient expression systems are particularly useful in the invention for expressing DNAs encoding amino acid sequence variants of rat ODF, to identify those variants that are functionally active.

Since it is often difficult to predict in advance the characteristics of an amino acid sequence variant of rat ODF, it will be appreciated that some screening of such variants will be needed to identify those that are functionally active. Such screening may be performed *in vitro*, using routine assays for receptor binding, or assays for osteoclast differentiation, or using immunoassays with monoclonal antibodies that selectively bind to rat ODF that is functionally active rat ODF, such as a monoclonal antibody that selectively binds to the active site or receptor binding site of rat ODF.

As used herein, the terms "transformation" and "transfection" refer to the process of introducing a desired nucleic acid, such a plasmid or an expression vector, into a host cell. Various methods of transformation and transfection are available, depending on the nature of the host cell. In the case of *E. coli* cells, the most common methods involve treating the cells with aqueous solutions of calcium chloride and other salts. In the case of mammalian cells, the most common methods are transfection mediated by either calcium phosphate or DEAE-dextran, or electroporation. Sambrook, et al., eds., *Molecular Cloning*, pp. 1.74-1.84 and 16.30-16.55 (Cold Spring Harbor Laboratory Press, 1989). Following transformation or transfection, the desired nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element.

Host cells that are transformed or transfected with the above-described plasmids and expression vectors are cultured in conventional nutrient media modified as is appropriate for inducing promoters or selecting for drug

resistance or some other selectable marker or phenotype. The culture conditions, such as temperature, pH, and the like, suitably are those previously used for culturing the host cell used for cloning or expression, as the case may be, and will be apparent those skilled in the art.

Suitable host cells for cloning or expressing the vectors herein are prokaryotes, yeasts, and higher eukaryotes, including insect, vertebrate, and mammalian host cells. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacillus* species such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for rat ODF-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*, Beach and Nurse, *Nature* 290:140-142 (1981), *Pichia pastoris*, Cregg, et al., *Bio/Technology* 5:479-485 (1987); Sreekrishna, et al., *Biochemistry* 28:4117-4125 (1989), *Neurospora crassa*, Case, et al., *Proc. Natl. Acad. Sci. USA* 76:5259-5263 (1979), and *Aspergillus* hosts such as *A. nidulans*, Ballance, et al., *Biochem. Biophys. Res. Commun.* 112:284-289 (1983); Tilburn, et al., *Gene* 26:205-221 (1983); Yelton, et al., *Proc. Natl. Acad. Sci. USA* 81:1470-1474 (1984), and *A. niger*, Kelly, et al., *EMBO J.* 4:475-479 (1985).

Suitable host cells for the expression of rat ODF also are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is useable, whether from vertebrate or invertebrate culture. It will be appreciated, however, that because of the species-, tissue-, and cell-specificity of

glycosylation, Rademacher, et al., *Ann. Rev. Biochem.* 57:785-838 (1988), the extent or pattern of glycosylation of rat ODF in a foreign host cell typically will differ from that of rat ODF obtained from a cell in which it is naturally expressed.

Examples of invertebrate cells include insect and plant cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. Luckow, et al., *Bio/Technology* 6:47-55 (1988); Miller, et al., in *Genetic Engineering*, vol. 8, pp.277-279 (Plenum Publishing, 1986); Maeda, et al., *Nature* 315:592-594 (1985).

Examples of useful mammalian host cells are the monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line 293 (or 293 cells subcloned for growth in suspension culture), Graham, et al., *J. Gen Virol.* 36:59-72 (1977); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells (including DHFR-deficient CHO cells, Urlaub, et al., *Proc. Natl. Acad. Sci. USA* 77:4216-4220 (1980); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980); monkey kidney cells (CV1, ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather, et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Construction of suitable vectors containing the nucleotide sequence encoding rat ODF and appropriate control sequences employs standard recombinant DNA methods. DNA is cleaved into fragments, tailored, and ligated

together in the form desired to generate the vectors required.

For analysis to confirm correct sequences in the vectors constructed, the vectors are analyzed by

5 restriction digestion (to confirm the presence in the vector of predicted restriction endonuclease) and/or by sequencing by the dideoxy chain termination method of Sanger, et al., *Proc. Nat. Acad. Sci. USA* 72:3918-3921 (1979).

10 The mammalian host cells used to produce the rat ODF of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, 15 Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham, et al., *Meth. Enz.* 58:44-93 (1979); Barnes, et al., *Anal. Biochem.* 102:255-270 (1980); Bottenstein, et al., *Meth. Enz.* 58:94-109 (1979); U.S. Pat. Nos. 4,560,655; 4,657,866; 4,767,704; 20 or 4,927,762; or in PCT Pat. Pub. Nos. WO 90/03430 (published April 5, 1990), may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts 25 (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics, trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an 30 equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for 35 expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure



encompass cells in culture *in vitro* as well as cells that are within a host animal, for example, as a result of transplantation or implantation.

It is further contemplated that the rat ODF of  
5 this invention may be produced by homologous recombination, for example, as described in PCT Pat. Pub. No. WO 91/06667 (published May 16, 1991). Briefly, this method involves transforming cells containing an endogenous gene encoding rat ODF with a homologous DNA, which homologous DNA  
10 comprises (1) an amplifiable gene, such as DHFR, and (2) at least one flanking sequence, having a length of at least about 150 base pairs, which is homologous with a nucleotide sequence in the cell genome that is within or in proximity to the gene encoding rat ODF. The transformation is  
15 carried out under conditions such that the homologous DNA integrates into the cell genome by recombination. Cells having integrated the homologous DNA then are subjected to conditions which select for amplification of the amplifiable gene, whereby the rat ODF gene amplified  
20 concomitantly. The resulting cells then are screened for production of desired amounts of rat ODF. Flanking sequences that are in proximity to a gene encoding rat ODF are readily identified, for example, by the method of genomic walking, using as a starting point the rat ODF  
25 nucleotide sequence set forth in Figure 1. Spoerel, et al., Meth. Enz. 152:598-603 (1987).

Gene amplification and/or gene expression may be measured in a sample directly, for example, by conventional Southern blotting to quantitate DNA, or Northern blotting  
30 to quantitate mRNA, using an appropriately labeled oligonucleotide hybridization probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-  
35 modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with

a wide variety of labels, such as radioisotopes, fluorophores, chromophores, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of the gene product, rat ODF. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu, et al., *Am. J. Clin. Path.*, 75:734-738 (1980). Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide based on the DNA sequences provided herein.

Rat ODF preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates. To obtain rat ODF that is substantially free of contaminating proteins or polypeptides of the host cell in which it is produced it is necessary to purify the rat ODF, based on the differential physical properties of rat ODF as compared to the contaminants with which it may be associated. For example, as a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. Rat ODF

thereafter is purified from contaminant soluble proteins and polypeptides, for example, by ammonium sulfate or ethanol precipitation, gel filtration (molecular exclusion chromatography), ion-exchange chromatography, 5 immunoaffinity chromatography, reverse phase HPLC, and/or gel electrophoresis.

Secreted, soluble rat ODF resulting from removal of the GPI membrane anchor acts is an example of an antagonist since it binds but does not activate its 10 receptor as demonstrated in the Examples. In contrast, secreted, soluble rat ODF IgG in which rat ODF extracellular domain is fused to an IgG Fc region acts as a rat ODF agonist. Its dimeric form may be responsible for its activity.

15 Amino acid sequence variants and derivatives of rat ODF are recovered in the same fashion, taking account of any distinguishing features or physical properties of the particular rat ODF. For example, in the case of a fusion protein comprising rat ODF and another protein or 20 polypeptide, such as a bacterial or viral antigen, a significant degree of purification may be obtained by using an immunoaffinity column containing antibody to the antigen. In any event, the ordinarily skilled artisan will appreciate that purification methods suitable for naturally 25 occurring rat ODF may require modification to account for changes in the character of rat ODF or its variants or derivatives produced in recombinant host cells.

The purity of rat ODF produced according to the present invention is determined according to methods well 30 known in the art, such as by analytical sodium dodecyl sulfate (SDS) gel electrophoresis, immunoassay, or amino acid composition or sequence analysis electrophoresis. Preferably, the rat ODF is purified to such an extent that it is substantially free of other proteins. Preferably, 35 the purified rat ODF will be greater than 99% rat ODF and, accordingly, non-rat ODF proteins will comprise less than 1% of the total protein in the purified rat ODF

composition.

Rat ODF may be used as an immunogen to generate anti-rat ODF antibodies. Such antibodies, which specifically bind to rat ODF, are useful as standards in assays for rat ODF, such as by labeling purified rat ODF for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or competitive-type receptor binding assays radioreceptor assay, as well as in affinity purification techniques. Ordinarily, the anti-rat ODF antibody will bind rat ODF with an affinity of at least about  $10^6$  L/mole, and preferably at least about  $10^7$  L/mole.

Polyclonal antibodies directed toward rat ODF generally are raised in animals by multiple subcutaneous or intraperitoneal injections of rat ODF and an adjuvant. It may be useful to conjugate rat ODF or a peptide fragment thereof to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (conjugation through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups.

Animals are immunized with such rat ODF-carrier protein conjugates combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with  $1/5^{\text{th}}$  to  $1/10^{\text{th}}$  the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-rat ODF antibody titer. Animals are boosted until the antibody titer plateaus. Preferably, the animal is boosted by injection with a conjugate of the same rat ODF with a different carrier protein and/or through a different cross-linking agent.

Conjugates of rat ODF and a suitable carrier protein also can be made in recombinant cell culture as fusion proteins. Also, aggregating agents such as alum are used to enhance the immune response.

5           Monoclonal antibodies directed toward rat ODF are produced using any method that provides for the production of antibody molecules by continuous cell lines in culture. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous  
10 population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. Examples of suitable methods for preparing monoclonal antibodies include the original hybridoma method of Kohler, et al., *Nature* 256:495-497 (1975), and the human  
15 B-cell hybridoma method, Kozbor, *J. Immunol.* 133:3001 (1984); Brodeur, et al., *Monoclonal Antibody Production Techniques and Applications*, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

          The monoclonal antibodies of the invention  
20 specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody  
25 class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired  
30 biological activity (Cabilly, et al., U.S. Patent No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci.* 81:6851-6855 (1984)).

          For diagnostic applications, anti-rat ODF antibodies typically will be labeled with a detectable  
35 moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may

be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g.,  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  
5 or  $^3\text{H}$ , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by David, et  
10 al., *Biochemistry* 13:1014-1021 (1974); Pain, et al., *J. Immunol. Meth.* 40:219-231 (1981); and Bayer, et al., *Meth. Enz.* 184:138-163 (1990).

The anti-rat ODF antibodies may be employed in any known assay method, such as competitive binding assays,  
15 direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987). Competitive binding assays rely on the ability of a labeled standard (e.g., rat ODF or an immunologically reactive  
20 portion thereof) to compete with the test sample analyte (rat ODF) for binding with a limited amount of antibody. The amount of rat ODF in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of  
25 standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

30 Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid  
35 support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David, et al., U.S. Pat No. 4,376,110. The second antibody

may itself be labelled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labelled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature references cited herein are expressly incorporated.

EXAMPLE 1                      CLONING AND SEQUENCING OF RAT ODF cDNA

A reverse transcriptase-PCR strategy was used to isolate rat ODF cDNA that encodes the functional domain. Degenerate oligonucleotide primers corresponding to the extracellular region of ODF were designed based on the published consensus peptide sequences from human and mouse (YFRAQM, AFKVR/QDID) (Lacey et al., 1998; Yasuda et al., 1998; Wong et al., 1997; Anderson et al., 1997). The primers used were as follows:

ODF sense

5' TA(C/T)TT(C/T)(A/C)G(G/A/T/C)GC(G/A/T/C)CA(A/G)ATG 3'                      SEQ ID NO:1

ODF antisense

5' (A/G)TC(A/T/G)AT(A/G)TC(A/G/T/C)(C/T)G(G/A/T/C)AC(C/T)TT(A/G)AA(G/A/T/C)GC 3'

SEQ ID NO.:2.

In view of the biological evidence for the bone loss in ovariectomized rats, the tibiae from these rats were selected as a source of RNA.

Total RNA was isolated from two tibiae from an ovariectomized rat. The tibiae were frozen in liquid nitrogen and kept in the -70°C until used. The frozen tibiae were ground into powder with mortar and pestle under

liquid nitrogen. 2 ml Solution D (4 M Guanidinium Thiocyanate, 25 mM Sodium Citrate (PH=7), 0.5% Sarcosyl, and 0.1 M 2-Mercaptoethanol) was added and the mixture vortexed thoroughly to break-up clumps of tissue. 400  $\mu$ l 2 M NaAc (pH=4) was then added, and the suspension was divided into 4  $\times$  2 ml aliquots and added to 2 microfuge tubes (about 0.7 ml each). An equal volume of phenol:CIAA (5:1) was added. The mixture was vortexed for about 30s, incubated on ice for 10-15 min and then centrifuged at 14000 rpm at 4°C for 15 min. The aqueous phase was transferred into a new microfuge tube and one volume of isopropanol was added. The mixture was precipitated at -20°C for 30 min and centrifuged as above. The supernatant was decanted, and all the pellets were combined and dissolved in 450  $\mu$ l 1  $\times$  TE and 50  $\mu$ l 3M NaAc pH=5.2. After addition of 500  $\mu$ l phenol:CIAA (1;1), the mixture was vortexed briefly and incubated on ice for 5 min and centrifuged as above. The aqueous phase was transferred into a new microfuge tube and re-extracted with 500  $\mu$ l CIAA. The RNA was precipitated from the aqueous phase with the addition of 3 volumes of 4 M NaAc (pH=7), and incubation at -20°C for 20min. After centrifugation, the RNA pellet was washed with 300  $\mu$ l 70% Ethanol, air-dried and dissolved in 20  $\mu$ l DEPC-treated water.

Single-stranded cDNA was then prepared from 2  $\mu$ g of total RNA using oligo-dT in 20  $\mu$ l of reaction. One-tenth of the reaction mixture was subjected to 2 cycles of PCR (94°C, 1 min; 37°C, 1 min; and 72°C, 2 min) in 1  $\times$  PCR buffer, 0.2 mM dNTP and 2.5 units of *Taq*. This was followed by 35 cycles of PCR (94°C, 1 min; 55°C, 1 min; and 72°C, 2 min). PCR reaction products were visualized on 1% agarose gels containing 0.5  $\mu$ g/ml ethidium bromide.

The resultant RT-PCR products corresponding to the predicted size of 747 bp were gel-purified using Gene-Clean™ method. Gel slices were dissolved in 3 volumes of 6 M sodium iodide (ICN Biomedicals Inc). 10  $\mu$ l of 50% glassmilk was added and the mixture was incubated on ice



for 10 min. DNA bound to the glassmilk was recovered after centrifugation (10 s, 12,000 RPM, at room temperature) and washed three times with ice cold washing solution (50% ethanol, 10 mM EDTA, 0.1 M NaCl, 10 mM TrisCl, pH7.5). DNA  
5 was eluted with 10  $\mu$ l of water and ligated into the pCR2.1 T/A cloning vector (Invitrogen). The ligation was conducted overnight at 16°C, followed by transformation into the Top10 bacterial strain (Invitrogen). Clones containing 747 bp predicted size of PCR products were  
10 selected for DNA sequencing. Sequencing was done by PCR-based method using D-Rodamine with M13 forward or reward primers under the following conditions (1  $\mu$ g of DNA, 1  $\mu$ l of 2  $\mu$ M primer and 4  $\mu$ l of premixed D-Rodamine in a final volume of 10  $\mu$ l). PCR was carried out using 96°C, 1 min;  
15 96°C 10 sec, 50°C 5 sec, 60°C 4 min; 60°C 1 min for 25 cycles. PCR products from sequence reactions were precipitated air-dried and subjected to automatic sequencing. One clone with striking sequence homology with human and mouse ODF was identified by sequence analysis and  
20 an homology search of the Genbank nucleotide database.

At the completion of the above experiment a rat ODF clone was identified; however, this clone only contained a portion of the rat ODF open reading frame. Accordingly, in order to identify the full-length open  
25 reading frame of rat ODF 5'- and 3'- end race strategy was employed.

To obtain 3'- and 5'-ends rat ODF coding sequence, 3'- and 5'-end amplification strategy was carried out as previously described (Frohman *et al.*, 1988).  
30 For 3' end race, first strand of cDNA was prepared from 2  $\mu$ g of total RNA using oligo-dT adaptor primer (5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTT 3') in 20  $\mu$ l of reaction. One-tenth of the reaction mixture was subjected to 30 cycles of PCR in 50  $\mu$ l of reaction (94°C, 1 min;  
35 54°C, 2 min; and 72°C, 2 min) using adaptor primer (5' GACTCGAGTCGACATCGA 3') and a rat ODF strand specific sense primer 1 (5' CAAGCCTGAGGCTCAGC 3'). 2  $\mu$ l of PCR product

was subjected to further amplification using the same condition with the adaptor primer and a rat ODF specific sense primer 2 (5' ACCAGCATCAAAATCCCA 3'). PCR products were ligated into the pCR 2.1 T/A cloning vector overnight at 16°C, followed by transformation into the Top10 bacterial strain (Invitrogen). ODF positive clones were identified using colony hybridization with a rat ODF 747 bp specific probe under stringent conditions and sequencing was carried out as above to obtain 3' end sequence of rat ODF ORF.

For 5'-end race, first strand of cDNA was prepared from 2 µg of total RNA using an ODF specific primer (5' GAACTTGGGATTTTGATGC 3') in 20 µl of reaction. The RT reaction was treated with RNase-H and passed through a microspin column (BioRad). 8 µl of this sample was used for dA tailing in 20 µl of reaction using the following conditions: 1 mM of dATP, 1 x buffer, 25 units of terminal transferase (Boehringer Mannheim), and 1.5 mM of CoCl<sub>2</sub>. The reaction was incubated for 15 min at 37°C and then 10 min at 65°C. 2 µl of dA tailing reaction was PCR-amplified using an ODF specific primer (5' TCGAGTCCTGCAAACCTG 3') and the oligo-dT adaptor primer in 50 µl of reaction for 30 cycles (94°C, 1 min; 54°C, 2 min; and 72°C, 2 min). 2 µl of the PCR products were subjected to 30 cycles (94°C, 1 min; 54°C, 2 min; and 72°C, 2 min) of PCR amplification using a rat ODF specific primer (5' GTGCTGTCTTCTGATATTCTG 3') and the adaptor primer. PCR products were ligated into the T/A cloning vector and sequenced as above to obtain 5' end sequence of rat ODF ORF.

Figure 3 shows the alignment of the deduced amino acid sequence of rat ODF, shown in Figure 2, with that of mouse and human. It can be seen that the rat has 84% sequence identity with human ODF and 96% with mouse ODF.

## EXAMPLE 2 TISSUE DISTRIBUTION OF RAT ODF

To examine the tissue distribution of rat ODF mRNA transcripts, PCR with rat ODF specific primers

followed by Southern blot hybridization with the rat ODF cDNA probe were carried out. Total RNA was isolated from various fresh-frozen tissues using RNAzol B solution strictly according to the manufacturer's instructions (Tel-  
5 Test, INC. USA), except RNA from bone tissues which was isolated according the protocol as above in example 1.

Single-stranded cDNA was prepared from 2 µg of total RNA using oligo-dT. 2 µl of each cDNA was subjected to 30 cycles of PCR (94°C, 40 sec; 54°C, 40 sec; and 72°C,  
10 40 sec) using rat ODF specific primers: ODF sense (5' aagctttGGATCCTAACAGAATATCAG 3') and ODF antisense (5' aagctTCAGTCTATGTCTTGAAC TTT 3'). As an internal control, the single stranded cDNA was PCR-amplified for 25 cycles using 36B4 primers (sense: 5' TCATTGTGGGAGCAGACA 3'; antisense:  
15 (5' TCC TCC GAC TCT TCC TTT 3') (Laborda J, 1991). RT-PCR products were run on an 1% of agarose gel and transferred on the Hybond-N<sup>+</sup> membrane (Amersham International). The membrane was hybridized overnight at 65°C with <sup>32</sup>P-labelled rat ODF cDNA or 36B4 cDNA probes respectively under  
20 stringent condition (3 x SSPE, 7%SDS). The membranes were washed 5 min with solution 1 (2 x SSC, 0.1% SDS) at room temperature, 20 min with solution 1 at 65°C, 20 min with solution 2 (1 X SSC, 0.1%SDS) at 65°C, 20 min with solution 3 (0.1 X SSC, 0.1%SDS) at 65°C. Each membrane was then  
25 exposed to X-ray film.

Figure 4 shows that expression of ODF mRNA was detected in thymus, rib crest, vertebrate, tibia, lung and spleen but not in brain, heart, kidney and liver. Radiographs showing the Southern blot analysis of the RT-  
30 PCR products of ODF (upper panel) and the control 36B4 (Laborda J, 1991) (lower panel) in various tissues. Lane 1, thymus; lane 2, lung; lane 3, rib crest; lane 4, vertebrate; lane 5, tibia; lane 6, liver; lane 7, spleen; lane 8, kidney; lane 9, brain; lane 10, heart.

35 In contrast, all samples expressed a similar level of 36B4. The level of ODF mRNA expression is highest in thymus, followed by bone tissues, spleen and lung.

To determine the size of the rat ODF transcript, Northern blot analysis was carried out with rat ODF cDNA as a probe. Given the more abundant expression of ODF mRNA in thymus and bone than in other organs, we selected thymus and bone tissues as sources of RNA for Northern blot analysis. 15  $\mu$ g of the total RNA was separated on a 1.2% agarose gel and transferred to Hybond-N<sup>+</sup> membrane (Amersham International). The membrane was hybridized for 15 h at 42°C with <sup>32</sup>P-labelled rat ODF cDNA probes. As an internal control, the membrane was rehybridized with a <sup>32</sup>P-labelled cDNA probe for rat 36B4. Each membrane was then exposed to X-ray film.

A single transcript with an approximate size of 2.4 kb was detected in thymus and rib crest.

15

#### EXAMPLE 3                      EXPRESSION AND PURIFICATION OF RAT ODF

Amino acid sequence analysis of rat ODF revealed that the structure of rat ODF is a type II transmembrane glycoprotein with a single hydrophobic region between residues 50 and 70. The hydrophobic sequence is likely to serve as transmembrane domain that separates ODF into a short N-terminal intracellular region and a long C-terminal extracellular region. The last 158 amino acid residues at the C-terminal region resemble  $\beta$ -sheet forming sequences of the TNF-like core and are predicted to function as the active ligand domain.

To express functional ODF proteins, cDNA containing amino acid residues from 160 to 318 or 246 to 318 of the ODF C-terminus were cloned into the bacterial expression vectors pGEX-3X and pGEX-2T, respectively (Smith and Johnson, 1988). CDNA was synthesized from 2  $\mu$ g of total RNA isolated from tibiae using oligo-dT in 20  $\mu$ l of reaction with moloney murine leukemia virus reverse transcriptase (Promega). 2  $\mu$ l of each cDNA was subjected to 30 cycles of PCR (94°C, 40 sec; 54°C, 40 sec; and 72°C, 40 sec) using rat ODF specific primers: ODF sense (5' aagcttTGGATCCTAACAGAATATCAG 3') and ODF antisense (5'

aagctTCAGTCTATGTCTTGAAC TTT 3'). The 732 bp rat ODF cDNA containing the extracellular domain was gel-purified and ligated into the pCR2.1 T/A cloning vector (Invitrogen). The resultant plasmid was designated pCR2.1-ODF2 which  
5 serves as a parental plasmid for the construction of ODF GST fusion protein. The pCR2.1-ODF2 plasmid was PCR-amplified with ODF specific primer (5' tctagatatCAAGCCTGAGGCTCAGC 3') and T7 primer (5' TAATACGACTCACTATAGGG 3'). The 480 bp PCR products were  
10 ligated into the pCR2.1 T/A cloning vector (Invitrogen) and the resultant plasmid was designated pCR2.1-ODF160A. A 480 bp *EcoRV* fragment from the pCR2.1-ODF160A was ligated into the *SmaI* site of pGEX-3T to make p3ODF1 which expressed the C-terminal region of ODF from amino acid residues 160 to  
15 318. The pCR2.1-ODF2 plasmid was PCR-amplified with ODF specific primer sense (5' agatctACCAGCATCAAAATCCCA 3') and antisense (5' agatctTCAGTCTATGTCTTGAAC TTT 3'). The 222 bp PCR products were ligated into the pCR2.1 T/A cloning vector (Invitrogen) and the resultant plasmid was  
20 designated pCR2.1-ODFB1. A 222 bp *BglIII* fragment from pCR2.1-ODFB1 was ligated to the *BamHI* site of pGEX-2T to make p2ODF1 which expressed the C-terminal region of ODF from amino acid residues 246 to 318.

Correct insertion of ODF cDNA in-framed with GST  
25 was confirmed by DNA sequence analysis and the resultant plasmid was named p3rODF1 and p2rODF1. To express GST fusion proteins, plasmids p2ODF1 and p3ODF1 were transformed into the bacterial strain Top 10' (Invitrogen) by heat shock, then plated onto LB plates containing 100  
30 µg/ml of ampicillin. To produce ODF GST fusion proteins, a single colony from the LB plate was inoculated into LB containing 100 µg/ml ampicillin and incubated overnight at 37°C with shaking. The next day, the bacterial culture was diluted 1/20 in fresh LB containing 100 µg/ml ampicillin  
35 and incubated at 30°C for 3 hours with shaking, IPTG was then added to the final concentration of 0.1 mM and incubation was continued for 4 hours at 30°C. Bacteria was

harvested by centrifugation at 5,000 RPM for 10 min at 4°C and lysed on ice for 30 min in a buffer containing 150 mM NaCl, 20 mM Tris-HCl and 1 mM EDTA. Triton X-100 was added to a final concentration of 1% and the bacterial lysate was  
5 subjected to mild sonication on ice for 2 min.

The resultant lysate was centrifuged of 10,000 RPM for 10 min at 4°C, and the supernatant collected. 1 ml of 50% glutathione agarose beads (Amersham Pharmacia Biotech) was added per litre of bacterial culture.  
10 Following incubation for 1 hour on ice, the glutathione agarose beads were collected and washed in phosphate buffered saline (PBS; pH 7.2) containing 0.1% Triton X-100 until no more protein was present in the washing solution as determined by spectrophotometry. GST-ODF fusion protein  
15 was eluted from the agarose beads with increasing concentrations of 10 mM, 20 mM, and 30 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted proteins were then analysed on a 10% SDS-PAGE to estimate the purity.

20 Fractions with GST-ODF greater than 95% pure were pooled and dialysed against 2 litres of PBS overnight at 4°C with one change. The protein concentration was estimated on a 10% SDS-PAGE against a serially-diluted bovine serum albumin (BSA) of known concentration (Promega)  
25 or quantitated by spectrophotometry.

GST-ODF was stably expressed as the predicted size of 46 kDa or 36 kDa protein. The expressed GST-ODF was partially soluble which allowed affinity isolation. The affinity-purified recombinant rat ODF proteins were  
30 greater than 95% pure.

#### EXAMPLE 4                      EFFECT OF RECOMBINANT RAT ODF IN VITRO AND IN VIVO

To examine the biological activity of recombinant  
35 rat ODF, we investigated the ability of ODF to induce osteoclastogenesis in spleen cell cultures.

3 x 10<sup>6</sup>/well spleen cells were cultured for 7

- 54 -

days in MEM containing 10% fetal calf serum (FCS) and 10 ng/ml of mouse macrophage colony stimulating factor (M-CSF) in the presence or absence of various concentrations (25 ng/ml to 250 ng/ml) of recombinant rat ODF in a 24 well plate. The cells were then fixed with 4% paraformaldehyde in PBS for 10 mins at room temperature and washed 4 times with PBS. The fixed cells were stained for tartrate-resistant acid phosphatase (TRAP) using Diagnostic Acid Phosphatase kit (Sigma) strictly according to manufacturer's instructions.

Figure 5 shows light microscopy images of the TRAP positive multinuclear cells from primary spleen cultured cells treated with ODF (left panel) or GST (right panel) in the presence of M-CSF. It can be seen that the addition of recombinant rat ODF at a dose of 30 ng/ml in combination of M-CSF to spleen cultures induced the formation of TRAP-positive multinuclear cells.

To test whether the multinuclear cells express calcitonin receptor, a marker of osteoclasts, RT-PCR was carried out using calcitonin receptor specific primer CR1a sense (5' TGGTGGAGGTTGTGCCCA 3') and CR1a antisense (5' CTCGTGGGTTTGCTCATC 3') at 60°C for 40 cycles (94°C, 40 sec; 60°C, 40 sec; and 72°C, 40 sec). Calcitonin receptor was expressed in spleen cells treated with rat ODF at a dose of 75 ng/ml in combination of M-CSF, but not with M-CSF alone or left untreated. These results demonstrated that recombinant rat ODF is capable of inducing osteoclastogenesis.

We found that recombinant rat ODF induced osteoclast formation in primary spleen cells. To further determine the effects of ODF on mature osteoclasts, we examined whether the ODF can cause polarization of osteoclasts *in vivo*. Two groups of rats were injected by subcutaneously either with 30 µg of GST-ODF or GST alone.

Tibias were also removed and blotted on glass slides. The cells were stained for TRAP activity, permeabilised and incubated with rhodamine-phalloidin. TRAP

expressing multinucleated cells were identified (Figure 7A and C), and filamentous actin visualised by confocal microscopy (Figure 7B and D). The total numbers of F-actin ring forming osteoclasts (polarised osteoclasts) and non F-actin ring forming osteoclasts were calculated under the  
5 florescent microscope.

Figure 8 shows that the percentage of TRAP positive osteoclasts with the presence of F-actin ring is significantly greater in rats that have received GST-ODF  
10 than rats that have received GST alone or nothing.

These results indicate that ODF stimulates mature osteoclasts to redistribute F-actin and to undergo polarization. Our F-actin ring assay provided evidence that more mature osteoclasts became polarized after the  
15 treatment of recombinant rat ODF. It is hypothesized that the increased percentage of polarized osteoclasts could result in increased bone resorption activity and thus increased level of blood calcium.

To test this hypothesis, blood was collected from  
20 individual rats 24 h after injection, and serum calcium concentration was determined. One day Wistar rats (n=6) were injected by the subcutaneous route with GST or recombinant rat ODF at a dose of 30 µg per animal. After 24 hours, serum was taken for the measurement of calcium.

25 As shown in Figure 6, the calcium concentration in rats injected with recombinant rat ODF was significantly higher than those injected with GST alone or not injected, indicating that recombinant rat ODF caused hypercalcaemia.

We have also shown that soluble ODF induces  
30 osteoclast formation in spleen cell cultures in the presence of M-CSF in a dose-dependent manner. The multinucleated cells induced by ODF are TRAP positive and capable of forming resorption pits on bone slices, and therefore satisfy major criteria of osteoclasts.

35 Recombinant ODF expressing the amino acid residues 160 to 318 is capable of inducing osteoclastogenesis, whereas recombinant ODF expressing



amino acid residues 246 to 318 do not exhibit functional activity, indicating that the TNF-like core domain is important for the function of ODF in osteoclastogenesis.

The polarization of osteoclasts is critical for bone resorption, which forms a tight seal with the substratum and results in an isolated resorption compartment. We have extended the functional analysis of rat ODF *in vivo* and shown that ODF induces polarization of osteoclasts and hypercalcemia. These results indicate that soluble ODF from the subcutaneous injection can circulate to the bone and induce osteoclast polarization. It has been shown that ODF can be cleaved when expressed in mammalian cells (Lum *et al.*, 1999; Lacey *et al.*, 1998). The cleavage of ODF occurred at amino acid residues 139 and produced a secreted functional ODF (Lum *et al.*, 1999). It is likely therefore that upregulation of ODF expression in other organs might have effects on osteoclastogenesis. This can be achieved through the shedding of ODF that then circulates to the bone to cause an effect.

#### EXAMPLE 5                      EFFECT OF RECOMBINANT RAT ODF ON THE TREATMENT OF NEW BORN RATS

To determine the biological effects of rODF (aa 160-318) *in vivo*, two day old Sprague Dawley rats (n = 6 per group) were injected by the subcutaneous route either with 30 µg of GST-rODF (aa 160-318) or GST alone. After 24 hours, serum from individual rats was taken for the measurement of calcium levels. Long bone tissues were also harvested from tibiae for histomorphometry and Rhodamine phalloidin and TRAP staining as previously described (Zheng *et al.* 1993; Yovich *et al.* 1998).

The numbers of TRAP-positive multinuclear cells *ex vivo* with or without the presence of F-actin rings were scored using confocal microscopy. As shown in Fig. 9A, injection of GST-rODF (aa160-318) induced hypercalcaemia in rats. The level of serum ionized calcium in GST-rODF (aa160-318) injected animals were significantly greater

than that in GST injected ones. It was also evident that the percentage of TRAP positive osteoclasts with a F-actin ring was significantly greater in rats which received GST-rODF (aa160-318) than those that received GST alone (Fig. 9 B&C). In Figure 9B the "a" shows the TRAP positive osteoclasts in newborn rats injected with GST-rODF (aa160-38), while "b" indicates the osteoclasts under the same field as 'a' display F-actin ring formation. The "c" indicates the TRAP positive osteoclasts in new born rats injected with GST alone, while "d" shows the osteoclasts under the same field as "c" do not display F-actin ring formation.

These results indicate that rODF stimulates polarization of mature osteoclasts. Further histomorphometry analysis revealed that the total number of osteoclast resorbing surfaces in GST-rODF (aa160-318) injected rats was significantly greater than that in GST injected rats. On the other hand, although the total numbers of osteoclasts per mm in GST-rODF (aa160-318) seems higher than in GST-injected animals, there is no obvious statistical significance between them (Fig. 9D). Taken together, these results demonstrated that the hypercalcaemia induced after 24 hours by the injection of rODF is most likely due to the induction of osteoclastic bone resorption.

Previous studies by Burgess et al (1999) and Lacey et al (1998) have demonstrated that RANKL/OPGL/ODF/TRANCE injection caused elevation of ionized calcium in the blood. The increase in ionized calcium in the blood was evident as early as 1 hr after the injection although frank hypercalcaemia was not observed until 2 days. The direct relationship of increased ionized calcium in blood with osteoclast activation has not been clearly defined. Gut calcium absorption seems unlikely to be the explanation for increased blood calcium levels (Burgess et al. 1999). In this study we have examined the polarization of osteoclasts *ex vivo* in rats injected with rODF. The

polarization of osteoclasts is critical for bone resorption, a process in which osteoclasts form a tight seal with the substratum resulting in an isolated resorption compartment. We have extended the functional analysis of ODF *in vivo* and shown that the TNF like core domain induces polarization of osteoclasts. The increase in ionized calcium in blood is co-incident with the induction of osteoclast polarization. Furthermore, histomorphometry analysis also demonstrated that rODF injected rats displayed high numbers of bone resorbing areas per bone surface whereas the total number of osteoclasts seems not to change significantly. Thus, based on the data, we suggest that ODF induced hypercalcaemia is at least in part due to the activation of existing osteoclasts at the bone surface (direct induction of osteoclast polarization).

REFERENCES

- Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D,  
5 Galibert L. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 1997 Nov 13; 390(6656):175-9.
- Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli  
10 C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S, Lacey DL 1999 The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 145:527-538.
- Frohman MA, Dush MK, Martin GR. Rapid production of full-  
15 length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci U S A* 1988 Dec; 85(23):8998-9002.
- Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms  
20 E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin  
25 ligand. *Proc Natl Acad Sci U S A* 1999 Mar 30; 96(7):3540-5
- Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli  
C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ,  
30 Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999 Jan 28; 397(6717): 315-23.
- Laborda J. 36B4 cDNA used as an estradiol-independent mRNA  
35 control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucleic Acids Res* 1991 Jul 25;19(14):3998.

- Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation Cell 1998 Apr 17; 93(2): 165-76.
- 10 Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP. Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and  
15 dendritic cell survival. J Biol Chem 1999 May 7; 274(19):13613-8.
- Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K. RANK is the essential  
20 signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun 1998 Dec 18; 253(2):395-400.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone  
25 density. Cell 1997 Apr 18; 89(2):309-19.
- Smith DB, Johnson KS. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 1988 Jul 15; 67(1):31-40.  
35
- Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y. TRANCE (tumor necrosis factor [TNF]-related

activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 1997 Dec 15;186(12):2075-80.

5

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS 3<sup>rd</sup>, Frankel WN, Lee SY, Choi Y. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997 Oct 3; 272(40):25190-4.

Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. Endocrinology 1998 Mar; 139(3):1329-37.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 1998 Mar 31; 95(7):3597-602.

Yovich S, Seydel U, Papadimitriou JM, Nicholson GC, Wood DJ, Zheng MH 1998 Evidence that failure of osteoid bone matrix resorption is caused by perturbation of osteoclast polarization. Histochem J 30:267-273.

Zheng MH, Fan Y, Wysocki S, Wood DJ, Papadimitriou JM 1993 Detection of mRNA for carbonic anhydrase II in human osteoclast-like cells by *in situ* hybridization. J Bone Miner Res 8:113-118.

## CLAIMS:

1. An isolated nucleic acid encoding a rat osteoclast differentiation factor (ODF) or biologically active fragment thereof.  
5
2. An isolated nucleic acid according to claim 1, wherein the nucleic acid is either genomic DNA, cDNA, RNA, or hybrid molecule thereof.  
10
3. An isolated nucleic acid according to claim 1, wherein the nucleic acid is a DNA molecule having:
  - a) a nucleotide sequence as shown in SEQ ID NO.:3; or
  - 15 b) a biologically active fragment of the sequence in a); or
  - c) a nucleic acid molecule which has at least 85% sequence homology to the sequence in a) or b); or
  - d) a nucleic acid molecule which is capable of  
20 hybridizing to the sequence in a) or b) under stringent conditions.
4. A rat ODF polypeptide or biologically active fragment thereof, wherein said polypeptide is encoded by a  
25 nucleic acid according to claim 1.
5. A polypeptide according to claim 4, wherein the polypeptide has an amino acid sequence as shown in Figure 2 or a biologically active fragment of said sequence.  
30
6. A polypeptide according to claim 4 or claim 5, wherein said polypeptide sequence is modified by one or more amino acid substitutions, deletions or insertions, or in the extent or pattern of glycosylation, but wherein said  
35 polypeptide substantially retains the biological activity of rat ODF.

7. A method of modulating the activity of cells comprising the step of administering to rat cells a protein encoded by a nucleic acid sequence according claim 1.
- 5 8. A method according to claim 7, wherein the activity modulated is selected from the group consisting of cell proliferation, cell differentiation and cell viability.
- 10 9. A process for the production of rat ODF comprising the steps of:  
growing under suitable conditions a microorganism transformed or transfected with a nucleic acid according to claim 1; and  
15 isolating the polypeptide product of the expression of said nucleic acid.
10. An antisense nucleic acid that is capable of binding to the coding sequence of rat ODF.
- 20 11. An antisense according to claim 10, wherein the antisense is complementary to a nucleic acid according to claim 1.
- 25 12. An antisense according to claim 11, wherein the antisense sequence inhibits the activity of rat ODF in cells when transfected into them.
13. An antisense according to claim 12, wherein the  
30 inhibition is selected from the group consisting of cell proliferation, cell differentiation and cell viability.
14. A fragment of rat ODF capable of eliciting an antibody which co-precipitates ligands that bind rat ODF.
- 35 15. An antibody elicited by a rat ODF fragment according to claim 14.



16. An antibody according to 15, wherein said antibody is produced by immunising an animal with rat ODF or a fragment thereof.
- 5 17. An antibody according to claim 16, wherein the rat ODF or fragment thereof is immunised in conjunction with an immunogenic polypeptide.
- 10 18. An antibody according to claim 15, wherein said antibody is a monoclonal antibody.
19. An antibody according to claim 15, wherein said antibody is a polyclonal antibody.
- 15 20. A polypeptide which is specifically co-precipitated by an antibody according to claim 15 from a cell expressing full-length rat ODF protein.
- 20 21. A polypeptide according to claim 20, wherein the cell is stably over-expressing the full-length rat ODF protein.
22. A polypeptide according to claim 20, wherein said polypeptide has the ability to bind to rat ODF, and capable of modulating an activity selected from the group consisting of cell cycle control, cellular differentiation and cell proliferation.
- 25 23. A method of screening for a ligand able to bind to and either activate or inhibit rat ODF, wherein said method is selected from the group consisting of immunoprecipitate with rat ODF antibody according to claim 15; screening lambda phage expression libraries for proteins that bind rat ODF peptides or fragments thereof; yeast two-hybrid system screening with fragments of rat ODF; solid-phase affinity binding assays using rat ODF
- 30 35

peptides and/or fragments and monoclonal antibodies to rat ODF and/or fragments thereof to compete for binding of rat ODF and epitope labelled rat ODF fragment screening for binding proteins in eukaryotic cell lysates.

5

24. A method of assessing the ability of a candidate compound to bind to a rat ODF comprising the step of:

incubating the rat ODF with the candidate compound under conditions that allow binding; and

10

measuring the bound compound.

25. A method of assessing the ability of a test compound to increase or decrease binding of an ODF to an ODF receptor comprising the steps of:

15

incubating ODF, ODF receptor and optionally the test compound under conditions that allow binding of the ODF to ODF receptor; and

measuring the binding of ODF to ODF receptor in the presence and absence of the test compound.

20

1/9

atgc

```

gccggggccaa ccgagactac ggcaagtacc tgcgcggctc ggaggagatg
ggcagttgcc ctggcgtccc acacgagggt ccgctgcac cgcgccttc
agcaccgggt ccagcgccgc ccccgccgc ctcccgttc atgttcctgg
cgctcctggg gctgggactg gtcagggtgg ctgcagcatc gctctgttcc
tgtactttcg agcgcagatG GATCCTAACA GAATATCAGA AGACAGCACG
CGCTGCTTCT ACAGAATTCT GAGACTCCGT GAAAATACAG GTTTGCAGGA
CTCGACTCTG GAGAGCGAAG ACACAGAAGC ACTACCTGAC TCATGCAGGA
GAATGAAACA AGCCTTTCAA GGGGCCGTGC AAAGGGAATT ACAACACATT
GTGGGGCCAC AGCGCTTCTC AGGAGTTCCA GCTATGATGG AAGGTTCTGT
GCTCGATGTG GCCCGGCGGG GCAAGCCTGA GGCTCAGCCG TTTGCTCACC
TCACCATCAA TGCTGCCGAC ATCCCATCGG GTTCCCATAA AGTCAGTCTG
TCCTCTTGGT ACCATGATCG AGGCTGGGCC AAGATCTCTA ACATGACGTT
AAGCAACGGA AAATAAGGG TTAACCAAGA TGGCTTCTAT TACCTGTACG
CCAACATTTG CTTCAGGCAT CATGAAACCT CAGGGAGCGT ACCTGCGGAC
TATCTTCAGC TGATGGTATA TGTCGTTAAA ACCAGCATCA AAATCCCAAG
TTCGCATAAC CTGATGAAAG GGGGGAGCAC TAAGAACTGG TCAGGGAATT
CTGAATTCCA CTTTATTCC ATAAACGTTG GAGGATTTTT CAAGCTCCGG
GCTGGTGAGG AAATTAGCGT CCAGGTGTCC AACCCTTCCC TGTTGGATCC
GGATCAAGAT GCGACGTACT TTGGGGCTTT CAAAGTTCAA GACATAGACT
GA

```

**FIGURE 1**

2/9

1	10	20	30	40
MRRANRDYGK	YLRGSEEMGS	CPGVPHEGPL	HPAPSAPAPA	
	50	60	70	80
PPPAASRFMF	LALLGLGLGQ	VVCSIALFLY	FRAQMDPNRI	
	90	100	110	120
SEDSTRCFYR	ILRLRENTGL	QDSTLESED	EALPDSCRRM	
	130	140	150	160
KQAFQGAVQR	ELQHIVGPQR	FSGVPAMMEG	SWLDVARRGK	
	170	180	190	200
PEAQPFahLT	INAADIPSGS	HKVSLSSWYH	DRGWAKISNM	
	210	220	230	240
TLSNGKLRVN	QDGFYYLYAN	ICFRHHETSG	SVPADYLQLM	
	250	260	270	280
VYVVKTSIKI	PSSHNLKGG	STKNWSGNSE	FHFYSINVGG	
	290	300	310	318
FFKLRAGEEI	SVQVSNPSLL	DPDQDATYFG	AFKVQDID	

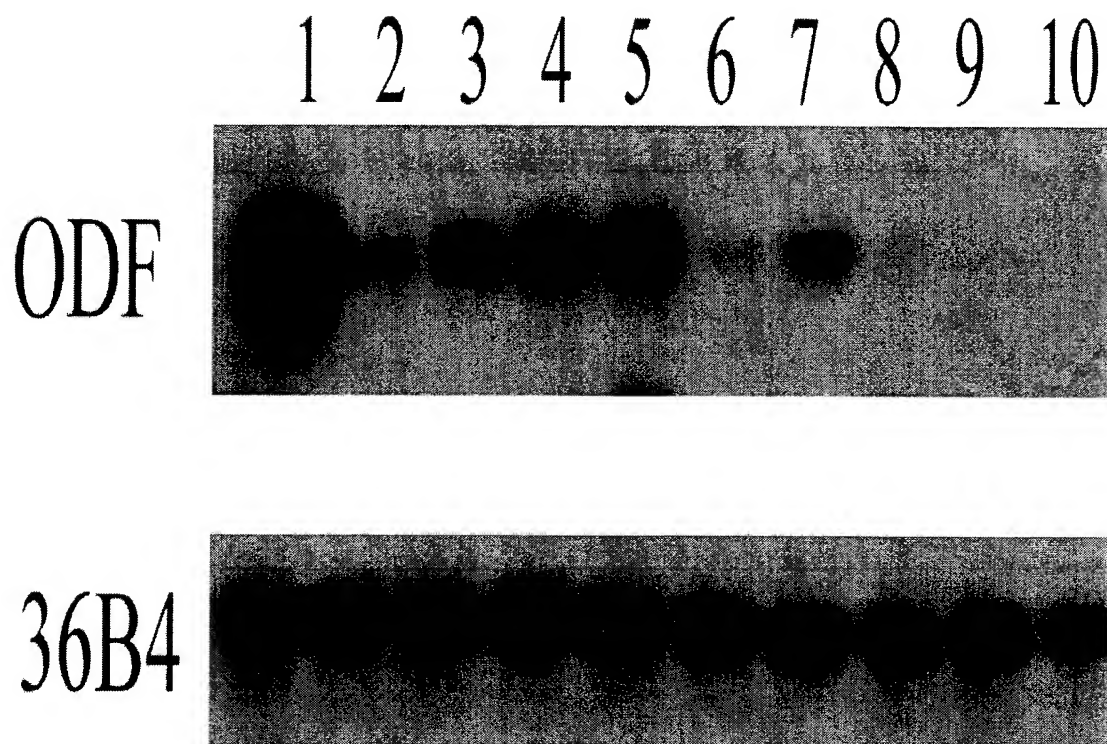
FIGURE 2

3/9

1	15 16	30 31	45 46	60 61	75 76	90
1 Human	MRRASRDYTKYLRGS	EEMGGPGAPHEGPL	H-APPPAPHPQPPAA	SRSMFVALLGLGLGQ	VVCSVALFFYFRAQM	DPNRISEDGTHCIYR 89
2 Mouse	MRRASRDYKGKYLRS	EEMSGPGVPHEGPL	HPAPSAPAPAPPPAA	SRSMFVALLGLGLGQ	VVCSIALFLYFRAQM	DPNRISEDSTHCFYR 90
3 Rat	MRRANRDYKYLRS	EEMGSCPGVPHEGPL	HPAPSAPAPAPPPAA	SRSMFVALLGLGLGQ	VVCSIALFLYFRAQM	DPNRISEDSTRCFYR 90
91	105 106	120 121	135 136	150 151	165 166	180
1 Human	ILRLHENADFQDTTL	ESQDTKLIPDSRRI	KQAFQAVQKELQHI	VGSQHRAEKAMVDG	SWLDLAKRSKLEAQP	FAHLTINADIPSGS 179
2 Mouse	ILRLHENAGLQDSTL	ESEDTEALPDSCRRM	KQAFQAVQKELQHI	VGPQRFSGAPAMMEG	SWLDVARRGKPEAQP	FAHLTINAASIPSGS 178
3 Rat	ILRLRENTGLQDSTL	ESEDTEALPDSCRRM	KQAFQAVQRELQHI	VGPQRFSGVPAMMEG	SWLDVARRGKPEAQP	FAHLTINAADIPSGS 180
181	195 196	210 211	225 226	240 241	255 256	270
1 Human	HKVSLSSWYHDSRGWA	KISNMTFSNGKLIVN	QDGFYLYANICFRH	HETSGDLATEYLQLM	VVVVTKTSIKIPSSHT	LMKGGSTKYWSGNSE 269
2 Mouse	HKVTLSSWYHDSRGWA	KISNMTLSNGKLIVN	QDGFYLYANICFRH	HETSGSVPTDYLQLM	VVVVTKTSIKIPSSHN	LMKGGSTKNWSGNSE 268
3 Rat	HKVSLSSWYHDSRGWA	KISNMTLSNGKLIVN	QDGFYLYANICFRH	HETSGSVPADYLQLM	VVVVTKTSIKIPSSHN	LMKGGSTKNWSGNSE 270
271	285 286	300 301	315 316	330 331	345 346	360
1 Human	FHFYSINVGFFKLR	SGEISIEVSNPSLL	DPDQDATYFGAFKVR	DID	317	
2 Mouse	FHFYSINVGFFKLR	AGEEISIQVSNPSLL	DPDQDATYFGAFKVQ	DID	316	
3 Rat	FHFYSINVGFFKLR	AGEEISVQVSNPSLL	DPDQDATYFGAFKVQ	DID	318	

FIGURE 3

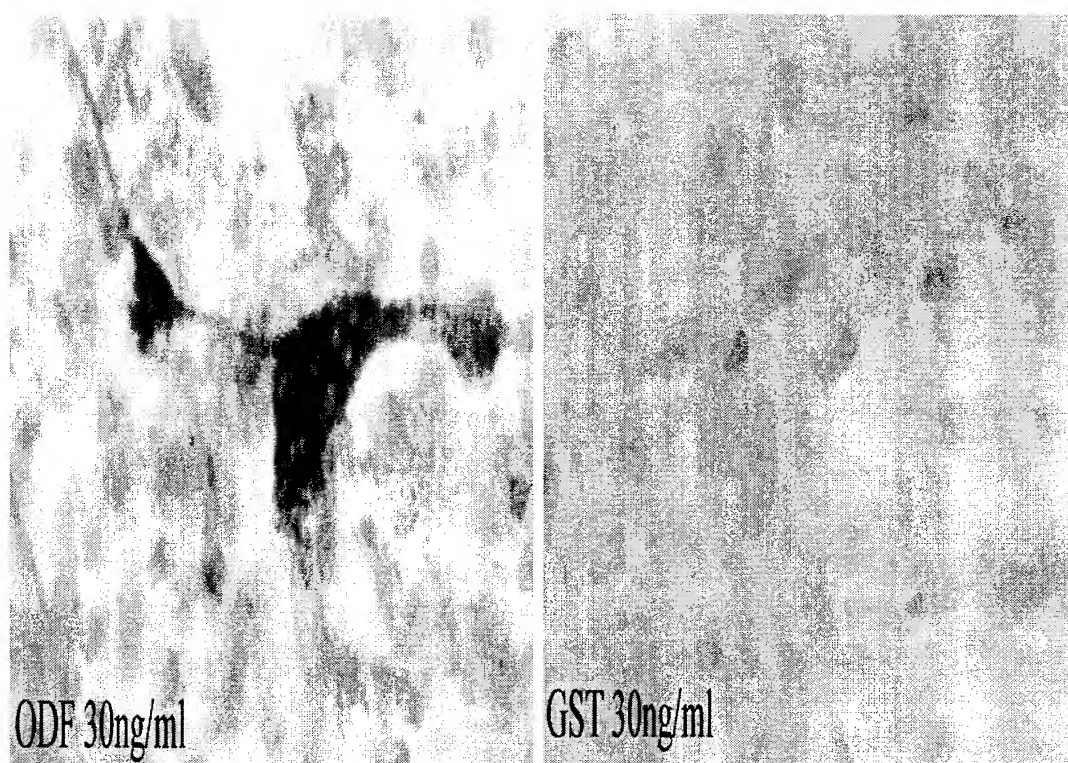
4 / 9



Tissue distribution of ODF mRNA

**FIGURE 4**

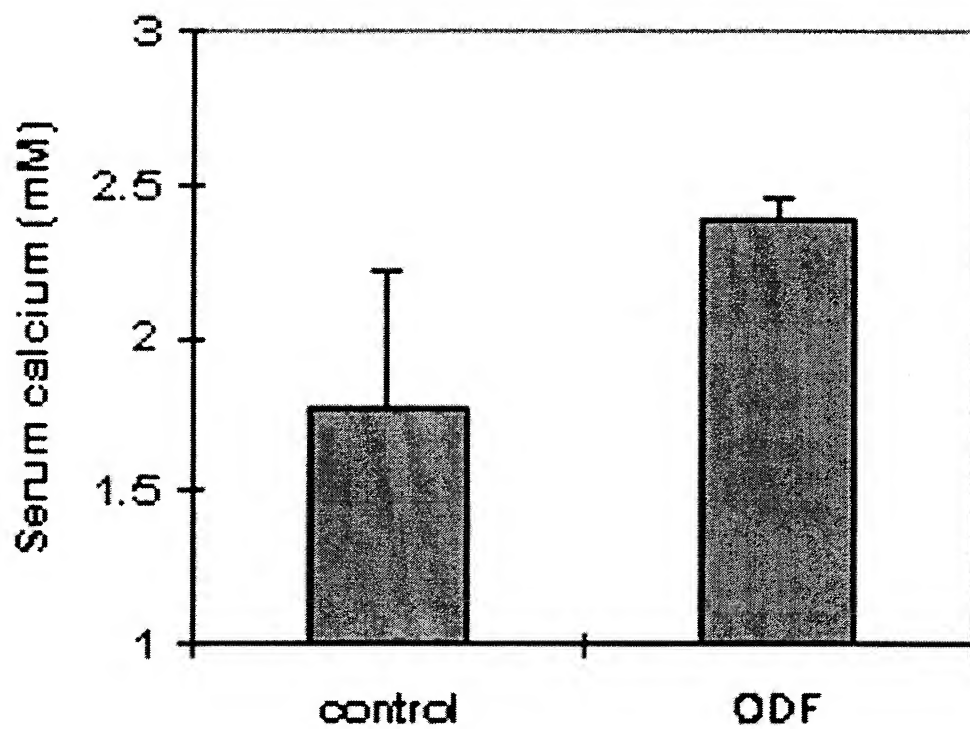
5 / 9



ODF induced osteoclastogenesis

**FIGURE 5**

6/9

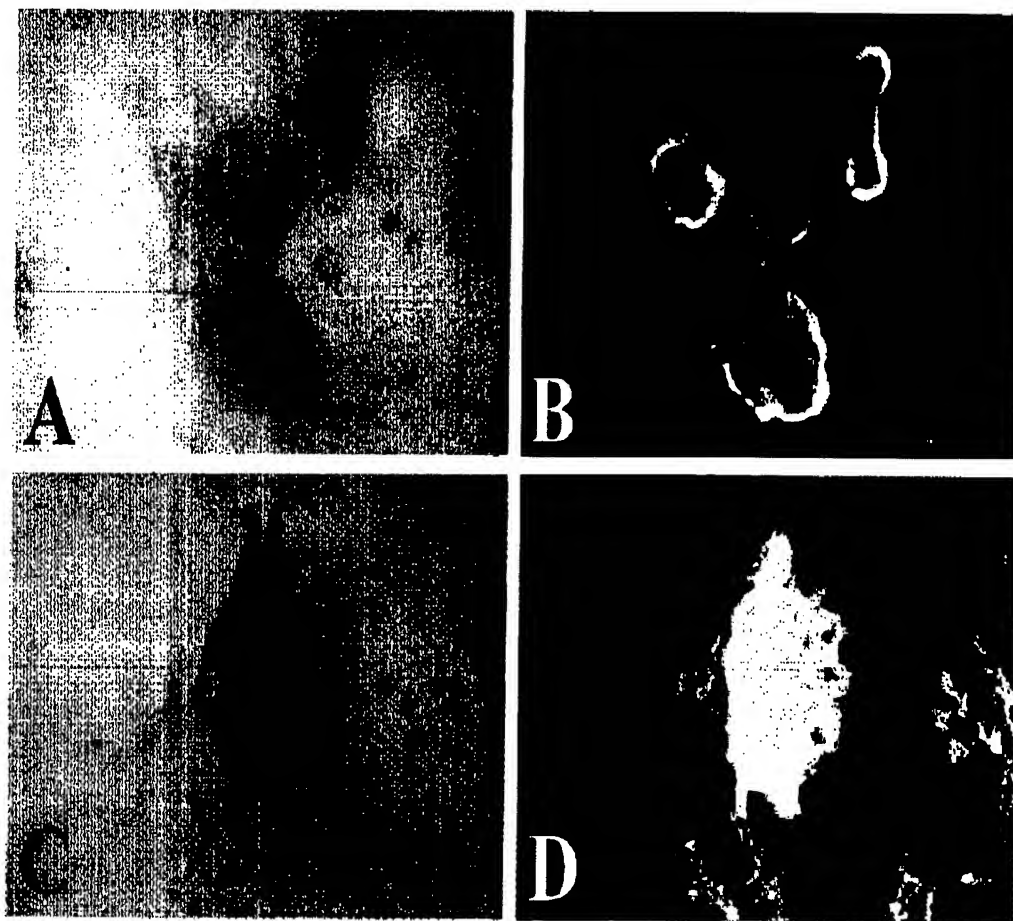


**ODF induced hypercalcemia**

**FIGURE 6**



7/9



**FIGURE 7**

8/9

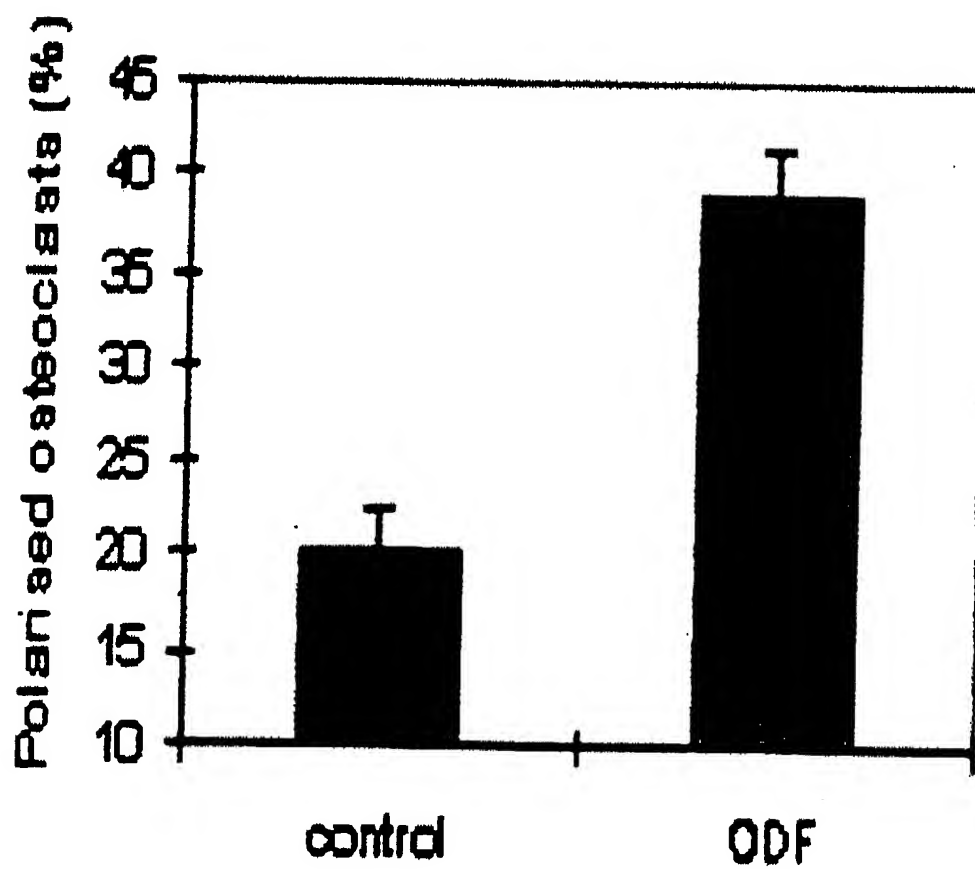
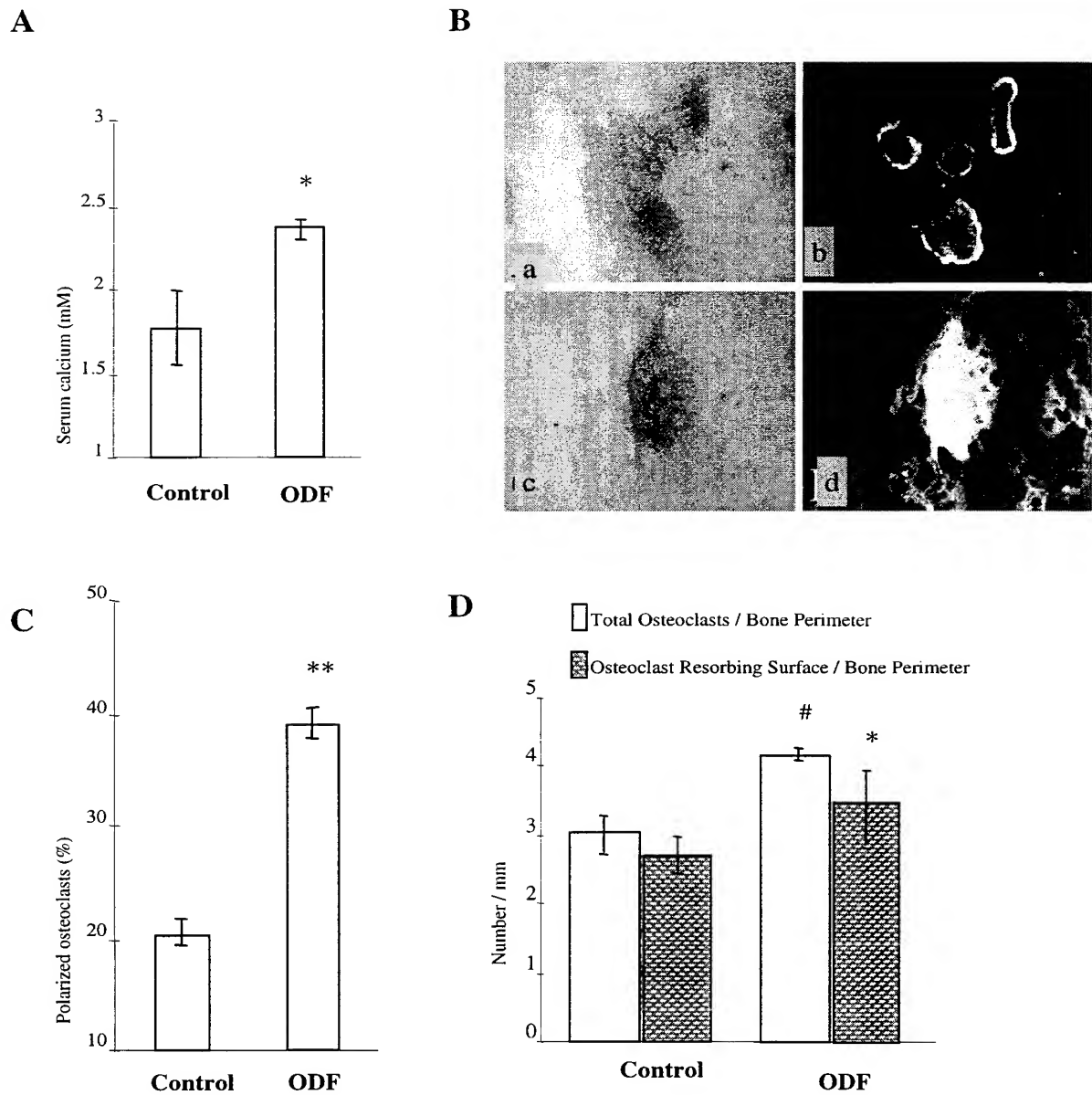


FIGURE 8

9/9

**FIGURE 9**

## SEQUENCE LISTING

<110> University of Western Australia

<120> Bone Cell Factor

<130> Zheng- Xu Application

<140> PCT/AU00/

<141> 2000-09-29

<150> PQ3147

<151> 1999-09-29

<160> 2

<170> PatentIn Ver. 2.1

<210> 1

<211> 957

<212> DNA

<213> Rattus sp.

<400> 1

```

atgcgcgcggg ccaaccgaga ctacggcaag tacctgcgcg gctcggagga gatgggcagt 60
tgccctggcg tcccacacga gggtcgcgtg catcccgcgc cttcagcacc ggctccagcg 120
ccgccccccg cgcctcccg cttcatgttc ctggcgctcc tggggctggg actgggtcag 180
gtgggtctgca gcatcgctct gttcctgtac ttctgagcgc agatggatcc taacagaata 240
tcagaagaca gcacgcgctg cttctacaga attctgagac tccgtgaaaa tacaggtttg 300
caggactcga ctctggagag cgaagacaca gaagcactac ctgactcatg caggagaatg 360
aaacaagcct ttcaaggggc cgtgcaaagg gaattacaac acattgtggg gccacagcgc 420
ttctcaggag ttccagctat gatggaaggt tcgtggctcg atgtggcccg gcggggcaag 480
cctgaggctc agcgttttg tcacctcacc atcaatgctg ccgacatccc atcggttcc 540
cataaagtca gtctgtcctc ttggtaccat gatcgaggct gggccaagat ctctaakatg 600
acgttaagca acggaaaact aagggttaac caagatggct tctattacct gtacgccaac 660
atltgcttca ggcacatga aacctcagg agcgtacctg cggactatct tcagctgatg 720
gtatatgtcg ttaaaaccag catcaaaatc ccaagttcgc ataacctgat gaaagggggg 780
agcactaaga actggtcagg gaattctgaa ttccactttt attccataaa cgttggagga 840
tttttcaagc tccgggctgg tgaggaaatt agcgtccagg tgtccaaccc ttccctgttg 900
gatccggatc aagatgcgac gtactttggg gctttcaaag ttcaagacat agactga 957

```

<210> 2

<211> 318

<212> PRT

<213> Rattus sp.

<400> 2

Met Arg Arg Ala Asn Arg Asp Tyr Gly Lys Tyr Leu Arg Gly Ser Glu  
 1 5 10 15  
 Glu Met Gly Ser Cys Pro Gly Val Pro His Glu Gly Pro Leu His Pro  
 20 25 30  
 Ala Pro Ser Ala Pro Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Phe  
 35 40 45  
 Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser  
 50 55 60  
 Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile  
 65 70 75 80  
 Ser Glu Asp Ser Thr Arg Cys Phe Tyr Arg Ile Leu Arg Leu Arg Glu  
 85 90 95  
 Asn Thr Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Glu Ala  
 100 105 110  
 Leu Pro Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val  
 115 120 125  
 Gln Arg Glu Leu Gln His Ile Val Gly Pro Gln Arg Phe Ser Gly Val  
 130 135 140  
 Pro Ala Met Met Glu Gly Ser Trp Leu Asp Val Ala Arg Arg Gly Lys  
 145 150 155 160  
 Pro Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Asp Ile  
 165 170 175  
 Pro Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg  
 180 185 190  
 Gly Trp Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg  
 195 200 205  
 Val Asn Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg  
 210 215 220  
 His His Glu Thr Ser Gly Ser Val Pro Ala Asp Tyr Leu Gln Leu Met  
 225 230 235 240  
 Val Tyr Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu  
 245 250 255

Met Lys Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His  
260 265 270

Phe Tyr Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu  
275 280 285

Glu Ile Ser Val Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln  
290 295 300

Asp Ala Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp  
305 310 315

## INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/AU00/01202****A. CLASSIFICATION OF SUBJECT MATTER**Int. Cl. <sup>7</sup>: C12N 15/12, 15/11, C07K 14/475, 14/51, 16/18, G01N 33/68.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

WPIDS (Derwent Dgene) Keywords - see selectronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
MEDLINE, GENBANK, EMBL, SWISS PROTEIN, PIR Keywords - see selectronic database box below.Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Sequence id nos 1 and 2.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	JP 2000102390 A (Y S NEW TECHNOLOGY KK and SNOW BRAND MILK PRODUCTS CO LTD) Derwent abstract accession No. 2000-332087/29, Class B04 D16, 11 April 2000. See sequence id nos 9 and 10 of the specification.	1-6, 9-14, and 20-25.
P, Y		15-19.
X,	Kodaira K <i>et al</i> "Cloning and characterization of the gene encoding mouse osteoclast differentiation factor" Gene vol 230 (1999) pp121-127 (1 April 1999). See the whole document especially the sequence in figure 3.	1-6, 9 and 20-22.
Y		10-19 and 23-25.
P, X	WO 2000-15807 A (M & E BIOTECH A/S) 23 March 2000. See the whole document especially sequence id nos 1 and 5.	1-6, 9 and 15-25.
P, Y		10-14.

☒ Further documents are listed in the continuation of Box C
☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 November 2000

Date of mailing of the international search report

23 November 2000.

Name and mailing address of the ISA/AU

 AUSTRALIAN PATENT OFFICE  
 PO BOX 200, WODEN ACT 2606, AUSTRALIA  
 E-mail address: pct@ipaustalia.gov.au  
 Facsimile No. (02) 6285 3929

Authorized officer

**J H CHAN**

Telephone No : (02) 6283 2340

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01202

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/29865 A (THE ROCKEFELLER UNIVERSITY) 17 June 1999	1-6, 9 and 10-22.
Y	See the whole document especially sequence id no. 3.	23-25.
X	WO 98/46751 A (AMGEN INC) 22 October 1998.	1-6, 9 and 10-25.
Y	See the whole document especially the sequences in figure 4.	
X	WO 98/46644 A (SNOW BRAND MILK PRODUCTS CO LTD) Derwent abstract accession No. 98-594563/50, Class B04 D16, 22 October 1998. See	1-6, 9 and 14-22.
Y	sequence id nos. 1, 2, 11 and 12.	10-13 and 23-25.
X	WO 98/28426 A (IMMUNEX CORPORATION) 2 July 1998.	1-6, 9 and 14-22.
Y	See the whole document especially sequence id nos 12.	10-13 and 23-25.
X	Yasuda H <i>et al</i> "Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to	1-6 and 9
Y	TRANCE/RANKL" Proc. Natl. Acad. Sci. USA, vol 95 pp3597-3602 (March 1998). See especially sequence disclosed therein (Genbank accession no. AB008426 and BAA25425)	10-25.
X	Wong B R <i>et al</i> "TRANCE is a novel ligand of the Tumor Necrosis Factor receptor family that activates c-Jun N-terminal kinase in T cells"	1-6 and 9.
Y	The Journal of Biological Chemistry, vol 272 no. 40 (October 3 1997) pp 25190-25194. See sequences disclosed therein (GenBank accession nos. AF013170, AF013171, AAC51762 and ACC71061)	10-25.
X	Anderson D M <i>et al</i> "A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function"	1-6 and 9.
Y	Nature vol 390 (13 November 1997) pp175-179. See sequences disclosed therein (GenBank accession nos. AF019047, AF019048, AAB86811 and AAB86812)	10-25.



International application No.  
**PCT/AU00/01202**

Patent Document Cited in Search Report			Patent Family Member				
JP	2000102390	NONE					
WO	200015807	AU	56173/99				
WO	9929865	NONE					
WO	9846751	AU	71205/98	BG	103824	BR	9808545
		EP	975754	NO	995044	PL	336311
		US	5843678	ZA	9803189		
WO	9846644	AU	68518/98	EP	911342	NO	985848
		ZA	9803159				
WO	9828426	AU	56180/98	AU	57184/98	EP	946725
		EP	951551	US	6017729	WO	9828424
END OF ANNEX							